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Resumos/Abstracts

Anti-tuberculosis drug resistance in Portugal

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Introduction: A sample survey of national drug resistance was undertaken included in the World Heath Organization's Global Project on Anti-Tuberculous Drug Resistance Surveillance, according to its defined protocol. About 1100 consecutive patients with smear positive pulmonary tuberculosis admitted to 46 randomly selected treatment centers were included in the survey from January 1995 to February 1998. The centers were randomly selected within each of the 18 administrative regions.

Material and Methods: Two sputum specimens per patient were sent to the National Reference Laboratory for Tuberculosis. Sputum specimens were cultured on Lowenstein-Jensen medium and Lowenstein-Jensen with pyruvate medium. The identification was based on culture aspects and standard biochemical tests. Susceptibility tests to four drugs: isoniazid, rifampicin, ethambutol and streptomycin were performed by the indirect proportion method. HIV testing was done by ELISA and confirmed by Western-blot. The Results are being evaluated and will be presented.

Incidence of *Mycobacteria* isolation in clinical samples from urinary tract during a 17–year period from Greece (1980-1996)

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Although pulmonary tuberculosis is the commonest localization, in recent years the incidence of extrapulmonary mycobacterioses seems to have been increasing mainly due to the immunocompromised patients.

The aim of the study was to determine the incidence of *Mycobacteria* isolation in urine from 1980 to 1996. 5152 urine specimens were cultured from equal number of patients and 182 *Mycobacteria* strains were isolated (the rate of positive cultures 3.5%). From 1980 to 1987, 36 of the 1575 patients who were examined, gave positive culture (2.3%). From 1988 to 1996, 146 of the 3577 patients who were examined, gave positive cultures (4.1%). From the 182 positive cultures, 144 samples were smear negative in Ziehl-Neelsen (79.1%) and 38 were positive (20.9%). 5 MOTT were isolated during the same period of time (4.3%): 2 MAC, 1 *M. chelonei*, 1 *M. ple*r and 1 *M. fortuitum*. From the 115 patients who had positive urine culture for *Mycobacteria*, 21 of them (18,3%) gave a positive sputum culture for *Mycobacteria*.

In conclusion, the incidence of *Mycobacteria* isolation in urine was noticeably significant during this long period of time. The coexistence of *Mycobacteria* in sputum in a remarkable number of patients is very important for the diagnosis of *Mycobacteria* infection.

Transmission of tuberculosis in Verona, Italy: a molecular study based on different typing methods

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Strain differentiation of *M tuberculosis* by DNA typing is an important component of tuberculosis surveillance in facilitating effective disease prevention and control measures.
Restriction Length Fragment Polymorphism (RFLP) analysis with the insertion element IS6110 is the most widely used genotyping method. However PCR-based typing techniques, such as Spoligotyping (Kamerbeek et al, Journal of Clinical Microbiology; 35; 4; 907-914)), have been developed to obtain a rapid differentiation of mycobacterial isolates. In this context Prod'hum et al. have recently described (Journal of Clinical Microbiology; 35; 12; 3331-3334) a ligation-mediated PCR (LMPCR) method for the amplification of flanking sequences located on the 5' side of IS6110.
The aims of our study are: i) to investigate the entity of tuberculosis transmission in an area of Northern Italy, the city of Verona and its countryside, where tuberculosis is endemic, ii) to evaluate the usefulness of the LMPCR as a rapid screening method for the fingerprinting of *M. tuberculosis* complex strains.
We studied 170 strains of *M.tuberculosis*, isolated from 168 patients at the Service of Microbiology of Verona, between 1 January 1996 and 31 December 1997. A computer-assisted analysis LMPCR was performed, and these data were compared with the Spoligotyping patterns of the same strains. All the strains included in clusters by one or both of these amplification-based typing methods were subjected to standard IS6110-RFLP fingerprinting.
The results and the conclusions of this study will be presented in further details.

Tuberculous neuromeningitis in Tunisia (257 cases)

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Objectives :
Review epidemiological, clinical and therapeutical profile and prognosis factors of tuberculous meningitis
Method and material :
In this retrospective multicentric study, the authors analyse 257 cases of tuberculous neuromeningitis, collected between 1974 and 1997. Diagnosis included tuberculous meningitis and encephalitic tuberculomes. Those cases were confirmed bacteriologically in 76 cases, histopathologically in 8 cases and presumed on other arguments in remaining cases.
Results :
Tuberculous neuromeningitis remain frequent in Tunisia despite a decrease of incidence in the last decade and accounts for 1 % of cases of tuberculosis. Most of the patients (56,5 %) were young less than 30 years old. 246 patients received antibacillary agents associated to corticosteroids in 171 cases. 4 patients underwent surgery. 91 patients (35,5 %) died after a prolonged coma. Recovery was achieved in 92 cases (35,8 %). 23 patients (8,9 %) still have neurological sequelae. 51 patients (19,8 %) were not revamped.
Poor prognosis was correlated with elderly, stage 3 of Gordon and Parsons, hyperalbuminorachia and delayed treatment.
Conclusion :
Diagnosis of tuberculous neuromeningitis was so difficult that a presumptive antibacillary treatment must be started early in this life-treatening infection. CTScan can help diagnosis and follow-up treatment.

Multicenter surveillance of *M. marinum* in Spain

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It has been carried out a survey of the isolation incidence of M. marinum on different Spanish cities to try get some knowledge of the frecuently isolted in each Hospital. The material consisted of data collected by a retrospective survey of the incidence of M. marinum in the following 21 hospitals of: Badajos, Barcelona, Córdoba, Granada, Madrid, Málaga, Murcia, Pamplona, Salamanca, San Sebastián, Santander, Sevilla, Terrasa, Valencia, Zamora. The methodology used was a retrospective epidemiological survey of all the laboratories in the cities mentioned above in order to obtain data on the incidence of M. marinum. Once the replies had been received, percentage incidence figures on the occurrence of M. marinum in the various Spanish cities were statistically and epidemiologically calculated. A total of 41 patients were detected between 1991 and 1998 (22 male and 19 female) the culture was made in 30 cases and the treatment was used usually Minocycline, Rifampin, Ethambutol and Charythromicin.

Molecular epidemiology of *Mycobacterium tuberculosis* strains isolated during one year in a French Department

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The aim of this study was to detect active tuberculous transmission and to identify the risk factors associated with the transmission of tuberculosis in an area with a low prevalence. Over one year (1997), we performed a systematic prospective survey based on the RFLP patterns of *M. tuberculosis* strains isolated from patients with tuberculosis diagnosed in Gironde (French department). Among the 132 studied strains, 110 were isolated from patients resident in Gironde, 22 in neighbouring department. DNA fingerprinting was carried out by standardized protocol of Van Embden. The RFLP patterns were scanned and compared by using the Gel Compar 3.1 software. A cluster was defined as two or more patients with identical RFLP patterns.
The number of IS6110 copies per isolate varied from 1 to 16 with a mean of 9 bands. Ninety nine patients (75%) were infected with genetically different isolates and 33 (25%) were grouped into 13 clusters of 2 to 3 identical clones. Compared with non clustered patients, clustered patients were younger (37 vs 48 years) and more likely to live in homeless shelters (3% vs 26%), in contrast, born in foreign country, HIV status, were not associated with clustering.
After comparison of information from interview and demographic data, among the 33 patients in clusters, epidemiologic connection to the other members of each patient's respective cluster were identified for only 12 patients (36%). For clusters without relation, a cross contamination of specimens in mycobacteriology laboratories cannot be suspected (dates and place of isolates) and nosocomial transmission was excluded after examination of hospitalisation period.

Mycobacterium SPP infection in three district councils of Setúbal – Portugal

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Oficial data show that the incidence rate of infection with *Mycobacterium* spp have been decreased in Portugal in the last five years.

Purpose: To study the microbiological and epidemiological data of *Mycobacterium* spp infected patients in the last five years, in 3 district councils of Setubal (Almada, Seixal and Sesimbra).

Methodes: Evaluation of the samples analised in the laboratory of Microbiology of the Hospital Garcia de Orta, Almada, between 1993 and 1997.

Each biological sample was processed as follows: 1º - Acid-fast stain with Kinyoun's carbolfuchsin and microscopic observation with a 100x oil immersion objective, 2º - inoculation in two Lowenstein-Jensen medium slants.

In the patients with positive samples in our laboratory between 1993 and 1997, we are studing the mycobacteriological results and the respective clinical process for: age, sex, race, adress, immune state, risk group, number and type of positive samples and laboratory vs clinical localisation of the disease.

Results: The results will be presented in the 19º Annual Congress of the European Society of Mycobacteriology, Lisbon, Portugal.

Mycobacterium tuberculosis complex DNA in naturally mummified individuals from 18 th century Vác, Hungary

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Several naturally mummified individuals were uncovered during reconstruction of a church in Vác, Hungary. The burials were in coffins stacked in layers and there was poor but continuous ventilation. An earlier initial study showed that there was evidence of tuberculosis in one of these individuals, as determined by CT and histology. Therefore, this and another individual were examined more fully, taking precautions against contamination of the material, and tissue samples were obtained from the lungs and abdominal cavity.

The tissues from the first individual were rehydrated using formal saline and embedded in paraffin wax. Sections were cut and stained with haematoxylin and eosin, toluidine blue, and Ziehl-Neelsen. The histology of the sections prepared from these tissues was surprisingly good and the basic architecture could still be seen in places. Many acid-fast bodies were visible both within the parenchyma and macrophages. These were the expected size of *M. tuberculosis* but cocco-bacillary in shape.

DNA was extracted from the samples using a modification of Boom's method[1] based on guanidium thiocyanate and silica. Taking stringent precautions against cross-contamination, a two-stage nested PCR was performed[2] using the Eisenach primers[3] for the IS*6110* sequence of the *M.tuberculosis* complex which results in a 92bp product. The tissue samples clearly yielded *M.tuberculosis* complex-specific DNA which supports the initial morphological findings.

The study of ancient DNA from microbial pathogens is of growing interest, as it enables the verification of traditional diagnosis, may answer long-standing questions in the history of disease, and, more excitingly, perhaps provide ancient DNA sequences that can be compared with those of modern pathogens.

(1) Boom, R. et al. J. clin. Microbiol. 28: 495-503, 1990.
(2) Taylor, G.M. et al. J. archaeolog. Sci. 23: 789-798, 1996.
(3) Eisenach, K.D. et al. J. infect Dis. 161: 977-981.

The epidemiological relatedness between two tuberculosis – explosions proven after eight years with the aid of DNA fingerprinting

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We describe the usefulness of spoligotyping, a novel and fast method of DNA analysis, to support epidemiological investigations for tuberculosis control.

In 1986 and 1994 two regional tuberculosis outbreak investigations were conducted. In 1994, spoligotyping is performed on dead bacteria from the suspected source from 1986, to analyse the chain of transmission.

The tuberculosis- explosions are limited to an island in the province of Zeeland and to an island of the noord-Holland area. The source patients of both explosions are identified in 1986 and 1994 respectively. Identical spoligotyping patterns of the source patients prove the transmission from a father to his son around 1986.

Thanks to the applicability of spoligotyping on non vital mycobacteria this DNA method contributes to retrospective epidemiological investigations. By performing spoligotyping directly on clinical material such as sputum, this technique might also improve prospective source case finding and contact tracing.

Correlation of mycolic acid detection with the incidence of tuberculosis in an archaeological population

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Globally, tuberculosis (TB) incidence is on the increase, the study of the disease in past populations may be of value in understanding the current rise. The study of the disease in past populations may be of value in understanding the current rise. Mycobacterial mycolic acids are stable 70 to 90 carbon 3-hydroxy, 2-long alkyl branched fatty acids they are major covalently bound lipids in the cell envelope of *Mycobacterium tuberculosis* and other mycobacteria. A preliminary study found TB mycolates in a 1900 year old medieval bone[1]. The former Newcastle Infirmary burial ground (AD1753-1845), excavated in 1997, provided a well-characterised skeletal population for study to evaluate mycolates as reliable biomarkers for ancient tuberculosis. The Infirmary records showed 27% of the cemetery population had tuberculosis at death, but osteological examination only showed 2.2% (0.95%) individuals with spinal changes consistent with TB (Pott's disease). Rib samples, chest cavity soil samples and distal soil samples were collected. Standard chromatographic profiles of mycolic acids were generated from *M. tuberculosis* and a range of possible contaminating mycobacteria, using a selective extraction protocol and high performance liquid chromatography (HPLC) analysis of methylanthryl esters with fluorescence detection. Archaeological rib samples and associated soils were processed in the same manner. The 24% of ribs positive for mycolic acids correlated with the documented 27% tuberculosis incidence. All the soil samples were negative.

1. Gernaey-Child, A. M. *et al.* (1998) Detection of mycolic acids confirms DNA evidence for tuberculosis in medieval human skeletal remains (submitted to *Proc. Natl. Acad. Sci.*).

Tuberculosis in AIDS patients. Drugs susceptibilities of *M. tuberculosis*

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Aim: to evaluate drug susceptibilities of *M. tuberculosis* (MT) isolated from AIDS patients (pts) and its relationship with risk factors, clinical presentation of tuberculosis (TB), and previous anti-tuberculous prophylaxis or treatment. Patients and Methods: retrospective study of the clinical records of HIV infected pts with the diagnosis of TB, based on the recovery of MT in organic fluids or tissues. MT was identified by conventional biochemical methods and/or by DNA hybridization (Accuprobe) from a culture in BACTEC or in solid media Lowenstein Jensen and Ogawa. One hundred and twenty three isolates of MT were tested for drug susceptibility studies. Demographic data were analysed. Results: a total of 136 pts was studied. Ages ranged from 19 to 58 years (x: 31.2±7.9); 107 (79%) were male. Risk factors elicited were IDU's in 102 (75%), heterosexuality in 20 (15%), homosexuality in 10 (7%) and transfusion or haemophilia in four (3%). Extra pulmonary TB was diagnosed in 79 (58%) pts, 56 with concomitant pulmonary involvement, and pulmonary TB in 57 (42%). TB was the AIDS defining opportunistic infection in 38 (27%). Drug sensitivity of *M. tuberculosis* is shown in the table.

	Isoniazid (INH)	Rifampin (RMP)	Pyrazinamide (PZA)	Ethambutol (EMB)	Streptomycin (STM)
Sensitivities	112/123	120/123	86/87	122/122	105/119
Resistant (% R)	11 (8.9)	3 (2.4)	1 (1.1)	0 (0.0)	14 (11.8)

Three IDU's MT was resistant to INH plus RMP and in one bisexual man MT was resistant to INH plus RMP plus PZA. These three unrelated cases occurred in 1997 and none had been submitted to chemoprophylaxis or previous treatment. Conclusions: Although multiple drug-resistance is still an unusual finding in our region, the existence of a large pool of IDU's pts is of major concern given their usual non-adherence to therapy - two out of the three cases were observed in IDU's. Rapid identification of MT is essential and susceptibility studies should always be carried out, in order to promptly institute proper infection-control procedures and adequate therapeutic regimens.

Transmission of tuberculosis in correctional facilities in Ile-de – France (France). A molecular epidemiological approach

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The transmission of Mycobacterium tuberculosis in correctional facilities presents a public health problem for correctional-facility employees, for inmates and for the communities into which they are released. We performed a prospective study in suspected tuberculous inmates in Ile-de-France jails. Seventy-three M. tuberculosis strains recovered from all the culture positive inmates during a three-year period (1995 to 1997) were studied by IS6110 RFLP standard analysis. Laboratory data and patient information files including information about incarceration were considered to exclude possible laboratory cross-contamination, to trace the intra-walls transmission of tuberculosis and to estimate the impact of the programs of prevention and control of tuberculosis transmission. Twenty-six isolates were clustered in 10 fingerprinting groups ranging from two to seven strains. Clusters included strains isolated along one or two years. The number of tuberculosis cases and clustered strains lowered along the three-year considered period. The risk factors for being included in a cluster are currently investigated.

Molecular epidemiology of bacillary tuberculosis in the Czech Republic

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In a prevalence study of 170 patients residing in Prague, West Bohemia (WB) and South Moravia (SM) resp. the fingerprinting method based on detection of the IS 6110 in the DNA of *M. tuberculosis* was applied. The RFLP profiles were divided into a group of non-clustered strains showing individually different RFLP pattern and a group of clustered strains displaying identical RFLP profiles.
In Prague 28% of strains fell into 7 clusters formed by 2-6 members, however, mutual contacts between patients in individual clusters were not established. In the WB and SM regions the proportion of clustered strains was 26 and 49% resp. and a few clusters were related to known family or community TB microepidemics. The prevailing proportion of non-clustered RFLP profiles suggests the endogenous reactivation of the persistent TB infection in a majority of patients, whereas the occurrence of clustered profiles seems to be related to recent exogenous transmission of TB. Higher age of non-clustered patients supports this hypothesis. As essential we consider the occurrence of the higher proportion of genetically identical *M. tuberculosis* clones circulating in the population of the SM region and participating in tuberculous microepidemics. These are apparently associated with a relatively low prevalence of TB which is the lowest in the Czech territory, e.g. twice as lower than in Prague.

Polymorphism in *Mycobacterium tuberculosis* complex isolates from Guinea Bissau

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Two hundred sixteen isolates of *Mycobacterium tuberculosis* complex from patients in Guinea Bissau were analysed for clonal origin by biochemical typing and by DNA restriction fragment polymorphism (RFLP) analysis using the insertion element IS6110.
The isolates could be divided into five distinct biovars, within a spectrum ranging from the classical human *M.tuberculosis* biovar to the *M.bovis* biovar. Upon RFLP analysis a significantly larger copy number of the IS6110 elements was found in the classical human *M.tuberculosis* biovar isolates, as compared to the intermediary biovars and the *M.bovis* biovar.
In terms of RFLP patterns and biochemical traits, there were two distinct groups of strains, one group comprising the majority of the classical human *M.tuberculosis* strains, with large numbers of fragments by IS6110 fingerprinting, and one group of strains mainly comprising isolates of the intermediary biovars and the classical *M.bovis*, with less complex fingerprinting patterns and fewer fragments by IS6110 fingerprinting.
It may be speculated that these two groups of strains have different origins, and may have evolved differently and independently in Guinea Bissau.

Epidemiological Surveillance in pulmonary tuberculosis

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By the end of the XX Century, Pulmonar Tuberculosis still affect wide the Portuguese population namely in the North with consequences in terms of morbility, mortality and absenteeism, having biz social repercurssion carried by these situations.
In Concelho of Vila Nova de Gaia it was felt need implemented a complementary program, in order to accomplish the "classic" program Obligatory Declaration Disease we felt the need to establish a program of Epidemiological Surveillance in Pulmonary Tuberculosis. The "classic" program appears to be insufficient for an adequate prevention and disease control. The best way to detect risks and establish strategies control for these infection goes necessarily through the intensification of epidemiogical surveillance, through improvement and development of infra-structures. These infra-structures are needed to recognise, notified and development an adequate aanswer of research used for the control of this disease and stress of it's intervention capacity.
One of the goals of this program was the set-up of a data base that allows an increase in the research, a better action and co-operation between the professionals. The goals of this program which started in June of 1997 were: create population data base; calculate the rates of prevalence, incidence and mortality; premature screening of the patients, adoption of adequate prevention measures and monitorization of multiresistant cases. The methodology used aimed to, besides, the set-up of data collection and registration supports at the Health Centres, Pneumological Diagnostic Centre and Hospitals, the data analysis and study with on time information and feedback. Since the beginning of it's set-up we managed to establish inter-institutional rules to a better disease control. We obtained therefore a joint action of the services for a better prevention, diagnosis, treatment and social reintegration.

Genetic differences and similarities among *M. avium* isolates infecting HIV – negative and – positive patients in Greece

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Forty-one human clinical isolates have been identified as *Mycobacterium avium* using PCR with the sets of primers AV6/AV7 and IN38 IN41. These *M. avium* isolates originated from 23 AIDS patients with disseminated disease, and 18 HIV-negative patients, of whom 11 children with cervical lymphadenopathy, five adults with lung infection, one with intra-abdominal infection and one with hip-joint infection. In order to examine the genetic diversity of isolates infecting HIV-negative and –positive patients, the strains were typed using pulsed field gel electrophoresis (PFGE) of genomic DNA following digestion with the restriction endonuclease *Xba*I. Random amplification of polymorphic DNA (RAPD) with primers A1245, B1245 & Leg1 was used as a second typing method. In the AIDS group, 10 different PFGE types could be distinguished, with approximately half the isolates clustering in one group. Similarly, 10 PFGE types were observed in the HIV-negative group, with approximately one third of the isolates clustering together. The predominant clusters in the two patient groups were genetically similar. RAPD analysis was largely consistent with these PFGE results. These results suggest that, though there exists genetic variability among *M. avium* isolates from HIV-negative and –positive patients, a predominant clone seems to account for a large number of infections in both groups in Greece.

Trend of *Mycobacterium tuberculosis* resistance to primary antituberculous drugs in Greece during a 17 – year period (1981-1997)

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The emergence of strains of *Mycobacterium tuberculosis* resistant to antimycobacterial agents is a worldwide problem whose magnitude is not well described.
The aim of the study was the surveillance of *M. tuberculosis* resistance to primary antituberculous drugs in Greece. 10267 strains *M. tuberculosis* were isolated from 1981 to 1997. Their resistance to streptomycin (STR), rifampin (RIF), ethambutol (EMB) and isoniazid (INH) was defined according to the proportion method. Table I show the rate of *M. tuberculosis* resistance to a single drug in two periods and Table II show the resistance to multiple drugs.

TABLE I

	1981-1990	1991-1997
STR	7,14	6,3
INH	8.71	6.0
EMB	3,79	2,6
RIF	7.59	2.0

TABLE II

	1981-1990	1991-1997
1 drug	6,72	5.36
2 drugs	3,45	1,95
3 drugs	2,14	1,05
4 drugs	1,27	1,08

From 1981 to 1997 the overall resistance of all the above drugs has declined. However it still remains high in comparison with other countries in Western Europe. In addition, in 1997 resistance rates had slightly increased, which means that their evolution needs to be kept under permanent observation.

Epidemiological study of tuberculosis in Brest suburbs

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We compared 40 clinical isolates collected from september 1995 to september 1996 by two methods in order to define the tuberculosis epidemiology of patients living in Brest surrounding. The methods used were Restriction Fragment Lenght Polymorphism of IS 6110, and AP-PCR (Arbitrarily-Primed PCR) using primers INS-2 and IS 986FP. Genomic DNA was prepared from cultures of M. tuberculosis isolates, digested by Pvu II and separated by electrophoresis on a 0.8% agarose gel. DNA was transferred to Hybond-N+ nylon membrane by Southern blotting and probed with a chemiluminescent IS6110 probe. AP-PCR procedure was as follow : 3 min at 94°C, 40 cycles (20 sec at 94°C, 1 min at 36°C, 1 min at 72°C), 7 min at 72°C. Amplified products were characterized by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.
Clinical data were obtained by review of patient's medical record with use of a standardized questionnaire.
The molecular typing data and the epidemiological findings were

compared statistically using a computerized multidimensional method : the factorial analysis of correspondence.
The typing results allowed us to identified several epidemiological clusters and to correlate the data obtained by conventional analysis.

Strains showed a high degree of variability in their *IS1245 RFLP* patterns with 30% of strains clustered into 5 patterns and 70% showing different patterns. Surprisingly however, 23% of the strains did not contain IS1245. These were mainly corresponding to isolates obtained from HIV negative patients.

* This research has been made within the GRUMAC working collaborative group.

A two year retrospective epidemiological study of drug – resistant tuberculosis in Germany using molecular methods

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In the last years, the treatment of TB has become complicated by the rising emergence of drug-resistant *Mycobacterium tuberculosis* strains. The proportion of drug-resistant strains in Germany remained stable at approx. five percent between 1992 and 1996. In recent years a rising number of epidemiological studies have been performed to elucidate the transmission dynamics of TB, especially to determine the importance of recent transmission compared to endogenous reactivation. These studies profit by modern molecular strain typing methods allowing an accurate identification of distinct *M. tuberculosis* strains and the determination of recent cases of transmission. In our study, we performed the first large-scale epidemiological analysis of drug-resistant TB in Germany using molecular methods. Approx. 400 *drug-resistant M. tuberculosis* strains sent to the National Reference Center in 1995 and 1996 have been analyzed with the *IS6110* fingerprinting method. Generally, a large degree of *IS6110* polymorphism was found, ranging from 1–20 copies. In both years approx. 30 % of all strains showed identical fingerprint patterns presumably representing recent cases of transmission. For some fingerprint groups transmission links could be confirmed by traditional epidemiological data. Beneath these fingerprint groups, three fingerprint families have been found showing very similar fingerprint patterns. The patients of these fingerprint families mostly are immigrants of the former SU. The biggest fingerprint family showed a similar *IS6110* and identical spoligotyping pattern as strains of the "Beijing family" which was found to represent a great proportion of *M. tuberculosis* strains in China and Asia. In conclusion, our results demonstrate (i) that transmission of drug resistant *M. tuberculosis* strains occurs and seems to contribute substantially to the emergence of drug-resistant TB in Germany, (ii) drug-resistant *M. tuberculosis* strains presumably are carried over from the former SU to Germany, and (iii) *M. tuberculosis* strains of the "Beijing family" could be found in Germany too.

Notification of tuberculosis cases from microbiology and histopatology departments in Emilia – Romagna region (Italy)

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TB incidence is underestimated in all European countries.Usually notifications are mandatory for clinicians only .
Since 1996 in Emilia Romagna Region (population 4 millions inhabitants)not only clinicians but also Microbiology and Histopathology departments must report TB cases to improve the quality of Tb surveillance, monitor new trends and detect unusual occurrence of the disease.
We report here the data collected in 1996 and 1997.
1996 reports from official notification system were 461 new cases, equal to 11.5 /100.000 inhabitants incidence.
1996 reports from Laboratory departments were 171 cases, of which 26 (18 from Microbiology and 8 from Histopathology departments) were unknown to surveillance system.
1997 reports from official notification system were 330 cases, equal to 8,2/100.000 inhabitants incidence.
1997 reports from Laboratory departments were 119 cases, of wich 11 (9 from Microbiology and 2 from Histopathology departments) unknown to the surveillance system.
Conclusions: Reports from Laboratories have improved the sensitivity of TB surveillances system in 1996 of 5.3% and in 1997 of 3.2%. The incidence per 100.000 inhabitants improved from 11.5 to 12.2 in 1996 and from 8.2 to 8.5 in 1997. Thus the new notification system have provided a better knowledge of the incidence of the disease in our Region.

Mycobacterium avium-complex (MAC) clinical isolates: drug susceptibility and RFLP typing of strains

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Several clinical isolates, identified as MAC by Gen probe, were obtained from HIV positive (44) and HIV negative (7) patients in two hospitals in Madrid (Spain) during 1996-1997.

Drug susceptibility of bacteria growing in 7H9 broth (Becton-Dickinson) were performed by testing six different drugs: Amikacin (4-8 µg/ml); Azithromycin (16-32 µg/ml); Clarithromycin (2-4 µg/ml); Ethambutol (4-8 µg/ml); Ofloxacin (2-8 µg/ml) and Rifabutin (2-5 µg/ml). DNA was obtained from colonies grown on Löwenstein-Jensen agar slants, and RFLP typing was performed using an internal fragment of IS1245 as a probe with non-isotopical methods for labelling and hybridization (Amersham).

Strains were mainly susceptible to all drugs. High similarities in the level of resistance/susceptibility were obtained in isolates from both hospitals. In particular, Azithromycin showed the highest level of resistance and Ethambutol the highest level of susceptibility.

Microbiological aspects of clinical isolates of *Mycobacterium avium-intracellulare* complex during years 1992 – 1997

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Over the past six years the number of clinical isolates of *Mycobacterium avium-intracellulare* complex obtained at our institution has increased constantly. Whereas numbers of positive blood cultures remained constant, increasing numbers of isolates have been obtained from the respiratory tract, pleural and joint fluid, bone marrow, lymph nodes, urine and stool. Blood and bone marrow samples have been cultured in bottles containing biphasic Middelbrook 7H9/7H10-LJ (Becton Dickinson, Milano Italy), while other samples have been cultured in solid and liquid media.
With regard to hospitalized HIV-infected patients we have found that positivity in a variety of body sites is consistently associated with blood culture positivity. In our experience, isolates from HIV-negative neoplastic patients have never been associated with blood culture positivity. Identification by species-specific DNA probes (Gen-Probe, San Diego, Ca.) revealed that 91% of isolates were represented by *M.*

avium, while only 9% of isolates were due to *M. intracellulare*. At the present time no different drug susceptibility patterns were found between strains of *M. avium* and *M. intracellulare*. Clarithromycin and ethambutol were the most effective drugs. Our experience indicates that in HIV-positive patients positivity for *M. avium-intracellulare* in any body sites should be further investigated by culturing blood and/or bone marrow.

Survey of national systems for drug resistance suveillance in Europe

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Objectives: As part of the EuroTB programme for the surveillance of TB in Europe, a survey was conducted in order to describe anti-TB drug resistance surveillance (DRS) activities conducted by European countries, to assess country adherence to the recently published WHO/IUATLD guidelines on DRS, and to evaluate the feasibility of establishing a European system for the collection and dissemination of DRS data.
Methods: A questionnaire was circulated among representatives of the 51 countries of the WHO European Region and, where relevant, among National Reference Laboratories (NRL).
Results: By April 1998, 27 questionnaires were obtained to EuroTB. Drug susceptibility testing (DST) is performed in all culture-positive TB patients in 19 of the 27 responded countries. The number of laboratories performing DST per million inhabitants range from 0.1 (United Kingdom) to 2.7 (France). NRL exist in 19/27 countries : 16/19 participate in the WHO/IUATLD proficiency testing scheme but only 8/19 organise DST proficiency testing at national level. National DRS is conducted by 17 countries - of which 10 can link laboratory data with clinical data of the TB notification - and regional or sentinel DRS by 9 countries. Primary and acquired resistance are evaluated separately in only half of the DRS systems described. Eighteen countries plan to implement or improve existing DRS in the near future.
Conclusions: Although DST is routine practice in most European countries, DRS providing results which are 1) representative 2) linked with valid clinical data and 3) evaluated with appropriate laboratory quality control is difficult to achieve. A European DST proficiency testing scheme as a component of the WHO/IUATLD network could help improving the validity of DST results. A European DRS data collection could be proposed base on minimum criteria for data quality.

Strains of *Mycobacterium tuberculosis* isolated in the South of Brazil: sensibility and polymorfism

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In Brazil, 35 to 45 million of people are infected with the bacillus of tuberculosis. Every year appear at this country about 110.000 new cases of this disease. The national incidence of tuberculosis is of 54,44/100.000, but its distribution is not homogeneous. This work studied 170 strains isolated of patients from Rio Grande do Sul, state of the South area, where the incidence is one lowest of the country. All the strains were studied to the test of sensibility with isoniazid (INH) and rifampicin (RMP) and 89 strains obtained randomically were molecular typed with the objective of evaluating the polymorphism.

The sensibility tests were accomplished by the method of the proportions and the polymorphism evaluation was accomplished by spoligotyping and RFLP. 34% of the studied strains, presented resistance at least to one drug. The largest resistance incidence was found in isolated of patients with history of previous treatment. In this group, 44%, were more than one drug resistant. Our results indicate a strong relationship between the resistance and the previous treatment. When studied under the molecular aspect, the strains showed polymorphism degree with RFLP larger than spoligotyping.

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Long-term trends in tuberculosis: comparison of age-cohort data in Hong Kong and England and Wales

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This study compared rates of tuberculosis, between 1954 and 1994, in different birth cohorts in Hong Kong with TB rates in similar birth cohorts in England and Wales, where the number of cases was around ten times lower. In both places, the rate of TB at any given age was lower for each successive birth cohort.

Age-specific TB rates in all birth cohorts showed a typical gradual decline in the probability of developing tuberculosis after the age of 24. After 1978 in Hong Kong and 1984 in England and Wales, rates of tuberculosis in all cohorts born before 1950 stopped declining and began to rise again, irrespective of their age at the time: the probability of developing TB began to rise after the age of 34 in the youngest cohorts (born 1940-49) but not until after the age of 60 in the oldest cohorts (born 1910-19).

Increasing risk of tuberculosis with age has never been observed before in previous cohort studies. The increase in different ages in different birth cohorts shows that increased longevity of the elderly today, with reactivation of latent disease, is not a major factor contributing to recent trends in tuberculosis. Higher levels of disease in immigrants of all ages with transmission of disease to all age groups could explain the ubiquitous increase in risk. However, better reporting of tuberculosis in recent years following increased global awareness may also be a contributing factor.

Surveillance of tuberculous meningitis in France in 1995. National Reference Center for Surveillance of Mycobacterial Diseases and Drug Resistance (NRCSMD), Paris, France

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To measure the incidence and describe the characteristics of bacteriologically confirmed tuberculous meningitis (TBM) in France, the National Reference Center for Surveillance of Mycobacterial Diseases and Drug Resistance conducted an active surveillance through a network of 375 microbiology laboratories. TBM was defined as a patient residing in France with a positive culture for tubercle bacilli of cerebrospinal fluid (CSF) taken from the 01.01.1995 to the 31.12.1995. The laboratories were asked to report all positive cultures of CSF along with identification and drug susceptibility test results, country of birth, age, sex, BCG vaccination and HIV co-infection of patients and outcome of treatment.
Responses were obtained from 370 of the 375 laboratories which reported a total of 49 TBM cases resulting in an overall annual incidence of 0.8 per million inhabitants (95 % CI = 0.6-1.1). Most cases (46) were reported by public hospitals and 22 (48 %) were from the two districts with the highest incidence of tuberculosis, Ile de France and Nord-Pas de Calais. Most patients (32) were adults over than 44 years and 33 (67 %) were French-born. HIV status was reported for 39 (79 %) patients among which 8 were HIV-positive. BCG vaccination status was known only for 10 patients, and 8 had been vaccinated. All isolated strains were *M.tuberculosis*. One was resistant to isoniazid alone and another to streptomycin alone. One year after diagnosis, 5 patients were lost to follow-up, 17 were dead and one had a relapse of tuberculosis. Among the 26 patients successfully treated, 12 had sequelae.

Among the 49 cases of TBM, 2 were from children under 5 years old giving an incidence of 0.55 per million (95 % CI = 0.07-2.0) in this age group. The 2 children had not been BCG vaccinated. The incidence in this age group is inferior to that observed in 1990 and the discontinuation of BCG vaccination in France may be discussed, except in the high risk groups.

will be needed to identify transmission sites and to the delineation of the epidemiologic links among patients, particularly in population sub-groups considered at risk to optimize the strategies to reduce transmission in the community.

Drug resistant tuberculosis in Slovenia from 1990 to 1997

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Drug-resistant tuberculosis represents a great problem in some parts of the world, but is assumed to be no problem for Slovenia. The aim of this study is to report about primary and acquired resistance to antituberculous drugs in Slovenia in eight years period.

More than 95 % of all susceptibility tests in Slovenia are performed in the Laboratory for Tuberculosis at the University Clinic of Respiratory and Allergic Diseases Golnik. In 2879 patients registered from 1990 - 1997 susceptibility tests on three to five first line antituberculous drugs were performed, using the method of proportion. One-hundred twenty-six patients (4.4 %) had drug-resistant tuberculosis. Multidrug resistance was present in 34 cases (1.2 %) in the same period.

All 2879 patients were verified by checking in the Central Register of Tuberculosis for Slovenia, Golnik, which started the registrarion in 1958. We identified 2370 patients who had never before been treated for tuberculosis. In 61 patients of this group (2.5 %) we found primary resistance to at least one antituberculotic. The percent of primary resistance oscillated from 1.5 % in 1993 to 3.5 % in 1992.

In conclusion, at present in Slovenia both the single-drug-resistant as well as multidrug-resistant tuberculosis remain low with no trend to increase.

Outbreak of multi-drug resistant tuberculosis in Lisbon metropolitan area

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An ongoing "Restriction Fragment Length Polymorphism" (RFLP) study of multi-drug resistant strains of *Mycobacterium tuberculosis* isolates from patients of several health institutions in the Lisbon Metropolitan Area revealed that the majority presented a single genetic pattern. In order to evaluate the existence of epidemiological links among this group of patients, we conducted a retrospective study based on the review of clinical records. Our study population consisted of a convenience sample of 27 patients, the majority of whom were HIV$^+$ drug addicts. Analysis of clinical records from the health institutions where these patients were treated revealed a multiplicity of potentially contaminating contacts. The existance of various epidemiological links among the patients, combined with data obtained by RFLP, suggests that an outbreak of nosocomially-transmitted multi-drug resistant tuberculosis has occurred. Given that in four cases, three of which were HIV$^-$ and without evidence of risk behaviours, no hospital contacts were identified, it is likely that this transmission is already taking place at the community level. Prospective epidemiologic studies using DNA fingerprinting techniques

Drug resistant tuberculosis in chronic excretors in Casablanca

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SETTING: This study was conducted at the Hospital 20 Août in Casablanca.
OBJECTIVES: To determine the nature of drug resistance among chronic patients with pulmonary tuberculosis and the polymorphism of strains isolated from these unrelated patients.
DESIGN: Prospective study.
METHODS: A total of 40 strains of M.tuberculosis isolated from 40 chronic cases were tested for susceptibility to rifampicin (RIF), isoniazid (INH), ethambutol (EMB), streptomycin (SM), ethionamid (ETH) and kanamycin (KAN).
The molecular typing of M.tuberculosis strains was performed by spoligotyping and RFLP analysis by using the IS 6110 probe.
RESULTS: Of the 40 M.tuberculosis isolates 70% were resistant to SM and RIF and 77% were resistant to INH and RIF (MDR-TB). IS 6110 DNA fingerprinting and spoligotyping showed identical patterns between six strains suggesting a local circulation of MDR-TB strains.
CONCLUSION: The high rate of MDR-TB in chronic cases was associated with incomplete anti-TB treatment. Urgent measures are needed to improve management of chronic cases and to limit spreading of MDR-TB strains.

Molecular epidemiology of multi-drug resistant (*MDR*) tuberculosis in Estonia

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Tuberculosis is a serious and increasing health problem in Estonia. Of special concern is the growing problem with multi-drug-resistant (*MDR*) tubercle bacteria. A population based study of TB drug resistance in Estonia in 1994 demonstrated that 10% of new and 19% of previously treated TB cases were infected with *MDR-TB* strains. These figures place Estonia among the countries with the highest *MDR-TB* rates in the world.

Since 1993 to 1997 we have studied the drug susceptibility pattern of Estonian *M. tuberculosis* isolates and examined them for clonal origin by analysing their restriction length polymorphism (*RFLP*) patterns using the insertion *IS6110* as probe.

Of new TB cases up to 29% have *M. tuberculosis* strains resistant to one or more of the drugs rifampicin, isoniazid, streptomycin and ethambutol, and about 11% have multi-drug-resistant (*= resistant to at least isoniazid and rifampicin*) strains. Altogether 274 isolates were studied for clonal origin. Among the 130 drug susceptible isolates, 37% belonged to clusters that showed identical RFLP banding patterns. More than half of the isolates, 63% with any drug resistance and 80% of *MDR* strains, belonged to 3 clusters with either identical or very closely related (>90%) banding patterns. The largest cluster comprised 9 identical and 21 closely related strains. *MDR* strains with these banding patterns were found among the isolates from 1993 until 1997. These findings indicate that a small number of drug resistant *M. tuberculosis* clones are now rapidly spreading in Estonia.

Microbiological and molecular – genetic analysis of *M. tuberculosis* cultures isolated from ÒÂ patients from Russia

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We studied biological properties of *M. tuberculosis* (LÒÂ) strains isolated from chronic patients with pulmonary tuberculosis from Moscow and different regions of Russia (Caucasus, Ivanovo oblast, Nizhny Novgorod). During the tuberculosis epidemics and particularly the spread of multi-drug resistant strains (MDR) of LÒÂ, of particular importance is molecular epidemiology based on genetic fingerprinting of LÒÂ strains from different geographic zones. We analyzed 96 specimens of mycobacterial DNA isolated from 39 ÒÂ patients. Drug resistance testing of LÒÂ specimens was performed using the absolute concentration method. We studied multi-drug resistant LÒÂ cultures, including rifampycin- and isoniazid-I resistant ones (43%), cultures sensitive to major antituberculosis drugs (37%), and cultures resistant to streptomycin, kanamycin, and ethambutol, but sensitive to rifampycin and isoniazid (16%).
Molecular and genetic investigations included the IS 6110 RFLP analysis and demonstrated wide genetic diversity of clinical isolates of *Mycobacterium*. Using fingerprinting in rifampycin- and isoniazid-resistant strains we revealed 71,5% strains which referred to the previously characterized W strain family, of which most strains referred to the W148 genotypic variant. Of the strains sensitive to all antituberculosis drugs 64% referred to the 001 variant. Of the resistant strains, sensitive to rifampycin and isoniazid, 56% referred to the 001 variant.

Discrimination of multidrug resistant *Mycobacterium tuberculosis* IS6110 fingerprint subclusters by *rpoB* gene mutation analysis

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The resurgence of tuberculosis was exacerbated by an alarming emergence of *Mycobacterium tuberculosis* strains resistant to antituberculosis drugs.
Seventy seven multidrug *Mycobacterium tuberculosis* resistant strains (MDR-TB) isolated in Lisbon hospital units in the period of 1996 to 1997 were characterized at the molecular level, by performing the standardized *IS6110* fingerprinting by restriction fragment length polymorphism (RFLP) analysis. We were able to differentiate 4 clusters (cluster A, B, C and D) containing 11-13 (57 isolates), 9 (7 isolates), 16 (2 isolates) and 15 (3 isolates) *IS6110* copies, respectively. Cluster A could be divided into 3 subclusters (A1, A2 and A3) according the restriction pattern (11, 12 and 13 bands).
In order to check the meaning of the small variations in RFLP patterns in cluster A, we used a second genetic marker, the mutational sites analysis of *rpoB* gene. A 69-bp region of the gene *rpoB*, encoding the β subunit of RNA polymerase in *Mycobacterium tuberculosis* was sequenced and mutations responsible for the rifampin resistance phenotype were checked, in several strains representative of each identified subclusters. In subcluster A1, all strains presented a substitution of Asp to Val in codon 516; in subcluster A2 it was found a substitution of Ser to Leu in codon 531. In subcluster A3, 4 types of substitutions were detected: Ser_{531} to Leu, His_{526} to Asp, His_{526} to Arg and Leu_{511} to Pro. These results, in subcluster A3, may suggest the occurrence of evolutionary divergence at clonal level.
In conclusion, the analysis of *rpoB* gene mutations confirmed that cluster A is in fact divided in 3 different groups suggesting the occurrence of independent outbreaks.

Risk factors for and clinical outcome of patients with primary multi-drug -resistant tuberculosis in Estonia, 1998

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Purposes: Primary multi-drug-resistant (MDR) tuberculosis (TB) is a significant problem in Estonia, and indeed in many nations. Approximately 15% of new TB patients in 1996 in Estonia were infected with MDR-TB strains. The purpose of the study was to describe the potential risk factors for and the treatment and clinical outcome of patients with primary MDR-TB in Estonia.
Methods: Cases were patients without a prior history of TB registered between Jan '94 and Dec '96 and with pre-treatment TB isolates fully resistant to at least isoniazid and rifampicin. Controls were new TB patients with isolates fully susceptible to all drugs tested. Information on both cases and controls was obtained from medical charts and physician and patient interviews.
Results: Of 51 eligible cases during the study period, 44 (84%) were enrolled with 44 matched controls. Twenty-five (57%) cases and 33 (77%) controls were male. Twenty-four (66%) cases and 12 (28%) controls had a history of prior hospitalization. Six (14%) cases and 4 (9%) controls had held a job in the health care industry. Fifteen (35%) cases and 16 (36%) controls were unemployed prior to their diagnosis, and 17 (40%) cases and 18 (42%) controls had a reported history of heavy alcohol use. Fifty-four percent of cases and 65% of controls had documented HIV test results, all were HIV negative. Only 17(39%) cases had completed treatment and were smear-negative by 1/98, compared to 26 (60%) controls. Eight (19%) cases and 0 controls died within 6 months of treatment initiation (p=0.01).
Conclusions: Patients infected with MDR-TB were, however, more likely to die, to remain smear positive, and to have a progressively worse CXR despite treatment that usually included second-line drugs. This study provides evidence that HIV-negative patients infected with MDR-TB suffer a worse clinical otcome than patients with pan-sensitive TB.

Molecular epidemiology of multi-drug tuberculosis in Spain

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Recently outbreaks of multidrug-resistant tuberculosis (MDR-TB) have been reported in hospitals in different European countries including Spain. Objective: Use of MDR-TB database of multidrug-resistant *M. tuberculosis* isolated in Spain in order to surveillance the wide distribution and the transmission of MDR strains. Material and Methods: The strains resistant to at least INH and RIF from the two Mycobacterial References laboratories of The Carlos III Institute. Other strains were sent from laboratories from different parts of Spain. They were studied by molecular typing using standardised DNA fingerprinting and spoligotyping. The patterns were analysed by GelCompar software. Results: Transmission between HIV-infected patients of multidrug-resistant tuberculosis caused by *M. bovis* was detected. From January 1997, the systematic typing MDR-TB isolates from the two Mycobacterial References laboratories was started. From 1998 a project is ongoing to study all the MDR strains isolated in Spain. The MDR-TB fingerprinting results of the database will be send the results to Centro Nacional de Epidemiología stabilising an early warning surveillance system for MDR-TB in Spain. On 31st 1998, 118 *M. tuberculosis* and 32 *M. bovis* MDR strains analysed by RFLP and 148 *M. tuberculosis* and 112 *M. bovis* by Spoligotyping were included in the database. 104 cases of the *M. bovis* were involved in the recent outbreak reported of MDR-TB caused by *M. bovis*. Conclusion: The use of centralised DNA databases can help to rapidly identify the origin and transmission routes of MDR-TB.

Synthesis and antimycobacterial activity of neosynthesized 2-pyridincarboxamidrazone derivatives

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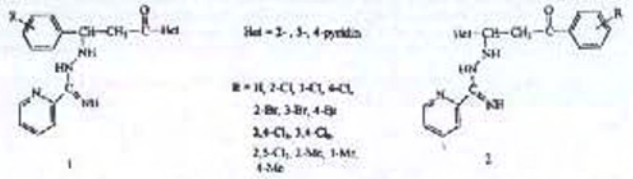
In previous papers (1-3) we described the synthesis and the antimycobacterial activity of some pyridine-2-carboxamidrazone and quinoline 2-carboxamidrazone derivatives. Some of these compounds showed interesting in vitro activity against some clinical strains of *Mycobacterium tuberculosis* isolated from human bronchial aspirates resistant to isoniazid, rifampicin and ofloxacin and against a human isolate of *M. avium*. We have synthesized a series of derivatives 1 and 2, containing a pyridine nucleus together with the 2-pyridincarboxamidrazone moiety, in order to evaluate their antimycobacterial activity.



1 2

In this work we tested by the agar dilution method the inhibitory activity of 21 different derivatives against 15 different human isolates of *Mycobacterium avium*: their MICs ranging from 4 μg/ml and 64 μg/ml.

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Antimycobacterial activity of rifabutin incorporated in conventional and long circulating liposomes

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M. avium, one of the microorganisms belonging to *M. avium* complex (MAC), is one of the most often pathogen isolated from AIDS patients. *M. avium* infection treatment remains a serious problem being still subject of clinical trials. The search for new drugs or drug carrier systems capable of penetrating efficiently into the infected cells is therefore essential. Among these carrier systems liposomes may be ideal vehicles for directing antibiotics to the cells of mononuclear phagocytic system (MPS) specially liver and spleen macrophages which represent the most important reservoirs of infections caused by *M. avium*. In fact, after intravenous administration, conventional liposomes composed of natural phospholipids are rapidly targeted to the cells of the MPS. Even the new generation of liposomes, characterized by the use of polyethylene glycol covalently bound to the phospholipid, although prolonging the circulation time of the liposomes in blood stream, eventually accumulate in infectious foci. So the aim of this work was to evaluate and compare the antimycobacterial activity of Rifabutin (RFB) liposomal formulations by using the conventional and the long circulating ones. This antibiotic, in the free form, is one antimicrobial agents that has been recommended by the U.S. Public Health Service as a prophylatic measure against infection due to MAC in patients with AIDS. The therapeutic activity of the developed liposomal formulations was evaluated in male Balb/C mouse-virulent infected with a *M. avium* strain. Treatment started two weeks after the infection, the animals received 3 injections a week during three weeks and two doses were tested: 10 and 20 mg/kg. The evaluation of *M. avium* growth in mice was determined by CFU countings in liver and in spleen previously homogeneized, serially diluted and plated on Middlebrook 7H10 agar. A strong reduction of the infection degree was observed for all the animals treated with the RFB liposomal formulations as compared to the untreated groups or to mice injected with RFB in the free form. The higher reduction was obtained for RFB formulations administered at a dose of 20 mg/kg. The use of conventional or long circulation liposomes (LCL), for the dose of 20 mg/kg, produced in the liver very similar reductions of the infection degree whereas for the spleen the use of LCL resulted in a higher reduction as compared to the conventional liposomes.

Antituberculosis drug toxicity in the pneumology department of Egas Moniz Hospital–Lisbon (3 years review).

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The authors conducted a retrospective study to evaluate the antituberculosis drug toxicity in the Pneumology Department of Egas Moniz Hospital over a period of 3 years (January 1995 to December 1997). From a total of 112 medical records of patients admitted with tuberculosis, 91 (81.25 %) were included in this study. 73 (80.22%) of these patients were male and 18 (19,78%) were female. Their median age was 38,57 years old (range: 17 to 84).
Initial drug regimens used were: H (isoniazid) + R (Rifampicin) – Z (pyrazinamide) + E (ethambutol) as 42 (46,15%) patients ; H+R–Z in 24 (26.37%) cases ; H+R+Z+S (streptomycin) in 19 (20.88%) patients and different other drug regimens in 6 (6.59%) cases.
Antituberculosis drugs toxicity was observed in 53 (58,24%) clinical settings. There were 21 (39,62%) patients with elevated serum uric acid concentration ; 13 (24,53%) cases of hepatotoxicity ; 2 (3,77%) cases of difficult serum glucose control in diabetics and 13 (35,13%) cases of multiple toxicity.
Most of the patients experiencing drug toxicity were asymptomatic (45 patients - 84,90%).
There was evidence of immunosuppression in 37 (69,81%) patients. HIV infection was the immunosuppressive disease most frequently found, diagnosed in 19 (54,35%) of the immunosuppressed patients.
In this study we draw the attention to the importance of antituberculosis drug toxicity with affected 53 (58,24%) of the patients studied and to the frequent association with immunosuppression.
Although the majority of the patients had mild toxicity, it is our impression that the near absence of clinical symptoms, in the setting of toxicity, is probably due to the fact that clinicians are conscious of antituberculosis drug toxicity, they easily monitor hospitalized patients and so toxicity is precociously diagnosed and treated.

Therapeutic efficacy of rifamycins in combination with interleukin 12 against *M. leprae* infection induced in nude mice

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In this study, we evaluated the efficacy of the combination of the benzoxazinorifamycin KRM-1648 or rifampicin with interleukin 12 (IL-12) against M. Leprae infection induced in athymic nude mice. BALB/c nude mice (female, 5 weeks old) were infected with 1×10^7 M. leprae Thai 53 in the hind footpads. KRM-1648 (0.6mg/kg) or rifampicin (6mg/kg) was given by gavage, once weekly, from days 61-150, and IL-12 (10μg/kg) was given ip, once weekly, from days 91-120. Ten months after infection, mice were killed and the numbers of acid-fast bacilli in both hind footpads were counted using the method of Shepard.

The anti-M. Leprae activities of KRM-1648 and rifampicin were increased when these agents were combined with IL-12, compared with each drug alone. Administrations of KRM-1648, rifampicin and IL-12 alone caused 1.6-, 2.1- and 0.3-log decreases in the number of leprosy bacilli/footpad, respectively, compared to those of control mice. The combinations of KRM-1648 + IL-12 and rifampicin + IL-12 caused 2.3- and 2.6-log decreases in the number of leprosy bacilli/footpad, respectively, compared to those of control mice.

The anti-leprosy activities of KRM-1648 and rifampicin were thus improved by combination with IL-12.

Is 6–month anti-tuberculosis chemotherapy enough for pulmonary tuberculosis in patients with diabetes mellitus?

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We conducted a retrospective cohort analysis to determine if 6-month short course chemotherapy (2HRZS or E/4HRE) for pulmonary tuberculosis in patients with diabetes mellitus(DM) is as effective as in those without DM. Sputum culture conversion rate, incidence of adverse reactions and the relapse rate after completion of 6-month regimen were compared. Fifty-six pulmonary new tuberculosis cases with DM completed 6-month regimen from January 1991 to December 1996. We selected for comparison two patients without DM who completed their first anti-tuberculous treatment in the same period per each patient with DM. These 112 patients were selected from a total of 282 cases and were matched for age, sex, and bacillary status at the beginning of chemotherapy. Sex ratio (M:F) in both group was 6:1, mean age of those with DM was 51.1 years-old and 50.9 years-old of those without DM. Ninety-six percent was culture positive in those with DM and 97 % was culture positive in those without DM. Nine percent of cases had some kinds of adverse reaction in both group. Conversion rate at two months of chemotherapy was 89%, and 93 % respectively. The relapse rate was 10.7 % (6 of 56) in patients with DM, and 1.8 % (2 of 112) in patients without DM (P<0.05). Adhesion to regimen in both group was good. We concluded that 6-month regimen for pulmonary tuberculosis in patients with DM was inadequate. These patients need more longer duration of chemotherapy, just as in HIV-infected tuberculosis patients.

In vitro activity of 7 disinfectants against mycobacteria other than tubercle bacilli

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Commercialized disinfectants must be submitted to official evaluation for testing their activity for disinfection procedures of surgical instruments against some infectious agents. Standardized procedures against mycobacteria include *Mycobacterium smegmatis* and optionally *M. tuberculosis*.
Atypical mycobacteria are largely present in the environment and may be agents of human infections. We tested the in vitro activity of 7 products, commercialized for the disinfection of surgical instruments, all containing glutaric aldehyde with different concentrations and formulae. The strains used for testing were isolated from biological specimens (fortuitous isolation) and were identified as *M. avium*, *M. fortuitum*, *M. gordonae*, *M. xenopi* and *M. terrae* by molecular hybridization to specific probes or by biochemical tests. Four disinfectants were used in our hospital these last years, one for dental instrumentation only. Products were tested following a procedure derived from AFNOR NF 72180, at the concentration and the temperature recommended by the manufacturer. All the products were tested for a 15 minutes contact time in the absence of interfering substance. The activity was considered good for a 6 log decrease of the initial concentration (CFU/ml) of the mycobacterial suspension.
Results show that 5 products have a good activity against *M. avium* and *M. gordonae* ; 4 against *M. terrae* and *M. fortuitum* and only 3 against *M. xenopi*. Three products only are active against all strains. The glutaric aldehyde concentrations of the latter products were 2% and 2 products contained also quaternary ammoniums. Among the 4 products selected by our hospital, only one had a good activity against all atypical mycobacteria in 15 minutes, the remaining three had a mean activity in 15 minutes which improved in 30 minutes. The data led us to modify some protocols.

Mycobacteria and safety in the laboratory

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Tuberculosis has always been at or near the top of the league of laboratory acquired infections.
There are two main modes of contracting the disease, the first, and most common, is by inhalation of aerosol droplets and the second by inoculation of tuberculous material.
Safety procedures are therefore designed to limit the production of aerosols and to prevent workers inhaling these droplets, and secondly to the production of a working method that eliminates accidental inoculations.
There are three steps towards the elimination of infection by aerosols - firstly the laboratory has to be designed so that the airflow within the room will remove any aerosols produced away from the workers. Secondly equipment has to be designed and used in such a way that any aerosols produced are entrapped and cannot reach the worker. Thirdly the worker has to be trained to work safely. This point is also fundamental in the elimination of accidental inoculations.
Overall once the laboratory has been designed properly, correct equipment supplied and safety procedures taught then the prevention of laboratory acquired infections is in the hands of the workers and their colleagues. A large number of infections can be prevented if the workers use common sense and do not alter standard practices. It is as easy to work safely as it is to work dangerously and the benefits are immense.

Sensitivity testing of mott strains by the MB/BACT

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Antimicrobial susceptibility of MOTT strains is difficult to predict. Therefore, rapid in-vitro-susceptibility tests are necessary for rational therapy of patients infected with these agents. Earlier, we have evaluated the MB/BacT (Organon Teknika) for susceptibility testing of *M. tuberculosis*. With the present study, we have expanded this work to include MOTT. Reference strains of *M. avium*, *M. fortuitum*, *M. marinum*, *M. kansasii* and others were tested for their susceptibility against INH, RMP, SM, EMB and when appropriate against ciprofloxacin (CIP), clarithromycin (CLA) and rifabutin (RBU). MIC values established with the MB/BacT were compared to results obtained with the Bactec 460. Both methods yielded identical results for 7 strains in 25 of 27 determinations. MIC determinations of CLA, RBU and CIP for 10 strains of *M. avium* were within one dilution step in 27 of 29 determinations. The remaining 2 determinations differed by 2 dilution steps. The study shows that in-vitro susceptibility tests can be reliably performed with the MB/BacT not only for M. tuberculosis strains but also for MOTT.

Susceptibility testing of *M. tuberculosis* by the MB/BACT (Organon Teknika) – a multicenter study

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A newly and extended multicenter study was performed for the evaluation of the susceptibility testing of M. tuberculosis (M.tb.) by the MB/BacT. 60 M.tb. patient strains were selected for the study, the frequency of drug resistance among these strains was as follows (data from Bactec 460 and proportion method, resp.): INH - 26, RMP - 15, SM - 19 and EMB - 8. Randomly numbered duplicates of the strains were distributed between 7 laboratories. The susceptibility testing with the MB/BacT was performed according to the earlier published procedure. Growth supplement containing oleic acid instead of tween was used. The test concentrations were (µg/ml): INH - 1, RMP - 1, SM - 1, EMB - 1 and 2, PZA - 50 (pH 5.5). The percentages of agreement between the MB/BacT results and the reference data were (sensitivity/resistance): INH - 99/100, RMP - 99/100, SM - 100/92, EMB 1 - 75/100, EMB 2 - 96/80. PZA was tested only for 3 resistant and 3 sensitive strains; 5 of 6 determinations were identical to the reference values. The data show that susceptibility testing by the MB/BacT is a real alternative to the established methods.

Reliability of MB/Bact culture system for testing susceptibility of *M. tuberculosis* to first line antibiotics

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OBJETIVE:
To evaluate the reliability of MB/Bact system (Organon Teknika) for testing susceptibility of *M tuberculosis* to Streptomycin (S), Isoniazid (I), Rifampicin (R) and Ethambutol (E) by comparing the results to those obtained by the radiometric BACTEC 460 TB system(Becton Dickinson).
MATERIAL AND METHODS:
A total of 30 isolates of *M tuberculosis* were evaluated including 4 control strains (*M tuberculosis* ATCC 35820, ATCC 35837, ATCC 35822, ATCC 35838). The concentrations tested were for MB/Bact: S (1µg/ml), I (1 µg/ml), R (1 µg/ml) and E (2 µg/ml) and for BACTEC, S (6 µg/ml), I (0.1 µg/ml), R (2 µg/ml) and E (7.5 µg/ml). An initial inoculum was prepared in a MB/Bact bottle. When the MB/Bact system detected the growth, 0.5 ml of the bottle were inoculated in each bottle containing the antituberculous drugs. Although an additional bottle with 0.5 ml of a 1/100 dilution of the inoculum was prepared. Times to detection (TTD) before the 1/100 dilution were considered resistant and TTD after 1/100 dilution were considered sensitive. The BACTEC TB susceptibility testing was performed according standard procedures.
RESULTS:
For 26 isolates, the results agreed by both methods to all antituberculous drugs tested. Only 4 discrepancies were observed: two isolates were susceptible to I in MB/Bact system but resistant in BACTEC system; One isolate was resistant to I in MB/Bact system and susceptible in BACTEC system; the last isolated was susceptible to R in MB/Bact system and resistant in BACTEC system. No discrepancies to S or E were observed.
CONCLUSIONS:
According to our results, the MB/Bact culture system appears to be reliable for testing susceptibility of *M tuberculosis* to first line antituberculous drugs.

Natural resistance to clarithromycin in *Mycobacterium tuberculosis* is effectively reversed by Sub-MIC concentrations of ethambutol

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Objectives: Investigate the concept that natural resistance to clarithromycin (CLA) in *M. tuberculosis* may be due to a permeability barrier located in the cell wall.
Materials and Methods: *M.tuberculosis* H37Rv and clinical isolates were selected for this study. Minimal inhibitory concentrations (MIC) of CLA and ethambuthol (EMB) were established using the BACTEC 460TB radiometric procedure. The combined action was performed in 12B media supplemented with half the MIC's of EMB and CLA. The drug combination data were based on X/Y quotient calculations.
Results: Exponential growth was observed in media containing either CLA or EMB, but was distinctly inhibited in vials containing both drugs. The data clearly demonstrated that sub-MIC concentrations of EMB were capable of reversing CLA resistance in the strains tested. Lack of reversal of CLA resistance was observed in an EMB resistant stain, as expected.
Conclusions: Sub-MIC concentrations of EMB reversed natural CLA resistance in *M. tuberculosis* EMB susceptible strains but not in an EMB resistant strain. This suggests that the concept demonstrated in *M. avium* attributing natural drug resistance to the organization of the cell wall outer layer also applies to *M. tuberculosis*.

Susceptibility tests of *M. tuberculosis* – radiometric and canetti methods

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A) - Material and Methods
40 susceptibility tests has been performed by radiometric method (Bactec) in strains of M. Tuberculosis and repeated with Canetti and coll method modified, when showing at least one resistance to four antibacillary antibiotics (Isoniazid, Rifampicin,Ethambutol and Streptomycin). In Canetti method we distinguished between no growth or Low-cention (named S) and a number of colonies between 0 and the critical proportion (named PS - poorly susceptible). Precision of each method has been evaluated.
B) - Results
We distinguished results from the first 13 tests (without final contamination control) from results with the following 13 tests (with final contamination control). Only the 27 tests with final contamination control were taken for comparison between radiometric and Canetti methods.
C) - Discussion and Conclusions
Concerning comparison between susceptibility tests with and without contamination control, disagreements were particularly relevant with Rifampicin and Streptomycin and most of them of the type resistant in Bactec-susceptible in Lowenstein. Such disagreements were much less obvious after contamination control. The Kappa statistic showed good concordance between Bactec and Lowenstein regarding INH (0.69 ± 0.13), Ethambutol (0.78 ± 0.15 with PS = R : 0.60 ± 0.21 with PS = S), and Streptomycin (0.68 ± 0.15 with PS = R and 0.77 ± 0.15 with PS = S) and very good with Rifampicin (1.0 ± 0.0 with PS = R and 0.85 ± 0.10 with PS = S). A significant difference was demonstrated between PS = S and PS = R with Rifampicin, in favor of PS = R, but no analogous differences were evident between Ethambutol and Streptomycin. McNemar test was non significant about difference between the 2 methods in Ethambutol (PS = R) and Streptomycin (PS = R or PS = S) cases.

Evaluation of MB/BACT for susceptibility testing of *Mycobacterium tuberculosis*

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Forty-two clinical isolates were included in this study. All strains underwent the BACTEC 460TB and the MB/BacT susceptibility tests, following the recommendations of the manufacturers. All resistant strains, which included the isolates with discrepant BACTEC and MB/BacT results, were tested by means of the conventional proportion method.

Test Drug	Both S	BACTEC S MB/BacT R	BACTEC R MB/BacT S	Both R
Isoniazid	30	0	0	12
Rifampin	30	1	1	10
Ethambutol	37	5	0	0
Streptomycin	31	1	1	9

Drug susceptibility test results by the two methods showed 95.8% overall agreement. The correlation between the two tests for resistant strains was 100% for for isoniazid (12 strains), 83.3% for rifampin (12 strains) and 81.8% for streptomycin (11 strains). A valid assessment of ethambutol was not possible due to the small number of resistant strains. The average reporting time were 9.1 days (range, 5.7 to 13.2 days) for MB/BacT and 9.2 (rage, 4 to 14 days) for BACTEC.

In our opinion, the main advantage of MB/BacT over the available BACTEC 460TB for susceptibility testing of *M. tuberculosis*, are its non-radiometric measurement principle and its fully automatization which implies that no action is required once the study is in motion.

Drug susceptibility test methods for rifapentine

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Rifapentine is a new rifamycin, approved in May 1998 by the Food and Drug Administration for tuberculosis therapy. The objective of this study was the development and justification of methods suitable for drug susceptibility testing of *M. tuberculosis* clinical isolates with rifapentine. Various laboratory strains, as well as 95 pansusceptible and 33 rifampin-resistant clinical isolates were tested, using agar- and broth-dilution methodology. Complete cross-resistance between rifapentine and rifampin was confirmed in this study. The MICs of rifapentine, when tested in 7H12 BACTEC broth, were in the range of ≤ 0.03 to 0.25 µg/ml for pansusceptible strains, and for only two of the 95 isolates the MIC was 0.25 µg/ml. The highest MIC of rifapentine when tested on 7H10 agar plates was also 0.25 µg/ml. The MICs of this drug for rifampin-resistant clinical isolates in broth were 8.0 µg/ml for 11, and greater than 8.0 µg/ml for 22 strains. On agar plates the MIC was 4.0 µg/ml for one strain, 8.0 µg/ml for four strains, and greater than 8.0 µg/ml for the remaining 28 resistant strains. The concentration of 0.5 µg/ml can reliably separate susceptible from resistant isolates, and therefore has been suggested as a critical concentration for broth methods. The advantage of the radiometric method in the BACTEC broth is the shorter turnaround time, less than two weeks for most isolates. The agar proportion method, with result available at three weeks, was more accurate in detecting drug resistance in strains containing less than 10% of resistant bacteria. Another advantage of this method is that it gives the actual proportion of resistant bacteria and has the option of being used as a direct test.

Drug susceptibility testing of *Mycobacterium tuberculosis* using MB/Bact® process system (Organon Teknika Corporation-Durham. USA)

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OBJECTIVE: To evaluate the usefulness and accuracy of *M. tuberculosis* drug susceptibility testing using MB/BacT, an automatic non-radiometric mycobacterial culture system.

DESIGN: First-line antituberculosis drugs susceptibility profiles for 70 strains of *M. tuberculosis* (45 of them known to be resistant to one or more drugs) isolated from different patients at our laboratory were determined. We used two standard reference methods (indirect proportion method on Lowenstein-Jensen medium and on Middlebrook M7H11 medium), as well as studying *M. tuberculosis* growth on MB/BacT vials with and without added drugs as recommended by the manufacturer.

RESULTS: We find good correlation in susceptibility/resistance patterns using MB/BacT system with results obtained by standard methods. The main advantages were: a) an important shortening in time required to detect drug-resistances and b) the system is easy to use and safe, as it requires manipulation of mycobacterial strains to a lesser extent than traditional methods, preventing cross-contaminations as well as infection hazards to staff.

Evaluation of the MB/Bact™ for drug susceptibility testing of *M. tuberculosis*

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OBJETIVE
To evaluate the MB/BacT ™ systeus as a method for *M. tuberculosis* susceptibility testing to Isoniazid (INH), Rifampicin (RIF), Streptomicyn (STR) and Ethambutol (EMB).
MATERIAL AND METHODS
We performed paired antimicrobial susceptibility test on 36 *M. tuberculosis* strains with MB/BacT ™ (Organon Teknika) and the BACTEC 460 TB (Becton Dickinson) wich represents the reference method for antimicrobial susceptibility test on mycobacteria. The tests were conducted according to the instructions given by the manufacturer.
RESULTS
The two methods showed concordance for the four drug tested in 32 cases (88,9%). There was disagreement in 2 cases (5,5%) for INH, 2 cases (5,5%) for EMB, 1 case (2,8%) for STR and 1 case(2,8%) for RIF. All the discrepancies were found in the resistant strains.
The mean time to reporting of drug susceptibility results was 7 days for MB BacT ™ verses 5 days for BACTEC 460 TB.
CONCLUSIONS
The MB/BacT ™ system is a good method for the *M. tuberculosis* sensitivity test without all the problems of radioactivity.

Comparison of clarithromycin-sensitive and clarithromycin-resistant *Mycobacterium avium* strains isolated from AIDS patients during therapy regimens including clarithromycin

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Sixteen *Mycobacterium avium* strains were isolated from the blood of 8 AIDS patients over a period of months. All the patients were on combination therapies including clarithromycin, and all had treatment failure and relapses of *M. avium* bacteremia. Paired clarithromycin-sensitive and -resistant *M. avium* strains isolated at the beginning of treatment and at the first relapse of bacteremia were compared.
When the *M. avium* isolates were hybridized with DNA probes specific for *M. avium* rRNA, we obtained lower hybridization values with clarithromycin-resistant isolates than with clarithromycin-sensitive isolates. This appeared to be due to smaller amounts of rRNA available for hybridization than to mutation of the 23S rRNA sequences in clarithromycin-resistant strains.
Random amplified polymorphic DNA analyses using 3 arbitrary primers showed that the clarithromycin-resistant isolates were clonally related to the clarithromycin-sensitive strains in 6 of the 8 patients. The other two patients had a polyclonal infection.
The *M. avium* isolates obtained on day 0 and after the emergence of resistance to clarithromycin did not differ in terms of their intracellular multiplication rate in human monocytes and J774 cells, or in terms of tumor necrosis factor alpha induction.
We infer that *M. avium* strains isolated during bacteremic relapses on combination therapies including clarithromycin are epidemiologically related to the initial strain and do not show changes in *in vitro* virulence. Polyclonal infections however, although less frequent, can also occur.

Susceptibility testing of *Mycobacterium tuberculosis* with ESP MYCO II system and MB Redox-System

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The ESP MYCO II culture system for susceptibility testing combines a liquid culture medium (ESP Myco) and a growth supplement (ESP Myco-GS) with a detection system that automatically incubates and continuously monitors culture bottles inoculated with specimens suspected of containing mycobacteria. The chemotherapeutic agents tested were isoniazid, streptomycin, ethambutol, rifampin and pyrazinamid. In our study we tested the time of *Mycobacterium tuberculosis* detection from culture. Moreover the ESP MYCO II system was compared to Bactec 460 TB with respect to the time and the results of susceptibility testing using 6 ATCC strains and 10 patient strains. Using ESP MYCO II, identical results to Bactec 460 TB were observed and the average time for detection was shorter.
MB Redox for susceptibility testing is a modified Middlebrook Medium combined with a colorimetric redox system. The chemotherapeutic agents tested were isoniazid, streptomycin ethambutol and rifampin. Using MB Redox, identical results to Bactec 460 TB were observed on ATCC strains and 10 patient strains. First growth detection with MB Redox was possible after 3 days and results remained unchanged for 10 days.

Resistance of clinical isolates of *Mycobacterium avium* to ethambutol, rifampin, streptomycin, ciprofloxacin and clarithromycin

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It has been always difficult to evaluate the correlation between the in vitro susceptibility of *Mycobacterium avium-intracellulare* complex and the clinical response to therapy. We have investigated the susceptibility of 43 isolates of *M. avium* obtained during 1997 from patients with neoplasia- or HIV-associated immunosuppression. Susceptibility tests were performed with an agar dilution method using both Middlebrook 7H10 and cation-supplemented Mueller-Hinton media. Cultures were performed in 35-mm dishes. Test drugs were used at the following concentrations: ciprofloxacin (1, 2, 4 μg/mL), clarithromycin (2, 4, 8 μg/mL), ethambutol (2, 4, 8 μg/mL), rifampin (0.5, 2, 8 μg/mL), and streptomycin (2, 4, 8 μg/mL).
All 43 strains had a MIC ≥4 μg/mL for ciprofloxacin (resistant). MIC_{90} could be obtained for: streptomycin (8 μg/mL; resistant), and ethambutol (4 μg/mL, intermediate). Though the MIC_{90} could not be determined for rifampin and clarithromycin, 30/43 strains (70%) had a MIC of 8 μg/mL for rifampin (resistant), whereas only 9/43 (21%) had a MIC of 8 μg/mL for clarithromycin (intermediate). In conclusion, in our series clarithromycin was the most effective drug in vitro, followed by ethambutol. Thirty-one out of 43 isolates (72%) were susceptible to both drugs. Though criteria for interpreting the in vitro susceptibility tests for MOTT have not been clinical infected patients have been treated according to the results of the above in vitro tests. This series of patients is currently followed for evaluating clinical conditions and survival times.

Infectiousness of *Mycobacterium tuberculosis* drug resistant (DR) and drud susceptible (DS) strains

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OBJECTIVES: to assess, over time, the infectiousness of *Mycobacterium tuberculosis* DR and DS strains.
METHODS: A 14 months cohort study of household contacts of DR and DS newly diagnosed active index cases of tuberculosis (TB) carried out in Santo Domingo, Dominican Republic. TB infection and active disease were investigated among the contacts. Tuberculin, Candida, and Tetanus Toxoid skin testing were applied at the initial visit and thereafter at 2 and 8 months of follow-up. Indurations > 5 mm and 10 > mm were used as cut-offs for positivity. Household contacts with features suggestive of TB were assessed for active disease by smear examination and radiographic methods.
RESULTS: Fifty-one DR index cases and 41 DS index cases were enrolled. 498 household contacts were visited 4 times. Overall, PPD indurations > 10 mm was detected in 154 (68%) of 227 contacts of DR index cases compared with 170 (64%) contacts of DS index cases ($p = 0.4$). The rate of tuberculin conversion at the follow-up visits was 22% in contacts of DR cases Vs 20% in contacts of DS cases respectively ($p = 0.5$). Active TB was diagnosed in 4% and 3% of contacts of DR and DS index cases ($p = 0.5$). No significant differences were observed with regard to the rate of infection and active TB among contacts of HIV-positive DR and DS index cases, nor among contacts of HIV-negative DR and DS index

cases. If a PPD induration > 5 mm was used as cut-off, contacts of HIV-negative DR index cases showed a significantly lower proportion of tuberculin conversion (17/82 [27%]) at the follow-up visits than contacts of HIV-negative DS index cases (41/87 [47%]) (RR = 0.6, 95% CI: 0.4, 0.9, p = 0.01).
CONCLUSIONS: These data show no differences in the rate of transmission of DR and DS strains with regard to TB infection (> 10 mm) and active disease. By decreasing the PPD cut-off to 5 mm, a lower rate of tuberculin conversion among contacts of HIV-negative DR index cases than that of contacts of HIV-negative DS index cases was found, no difference was observed, however, between contacts of HIV-positive DR and DS subjects. Analysis accounting for anergy and other factors is currently under evaluation.

Antituberculosis drug susceptibility test using MB/BACT culture system: first approach

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Objective: Develope a method for susceptibility testing of mycobacteria using the isotope-free liquid culture media MB/BacT (Organon Teknika Corp. U.S.A.).
Material and methods: A total of 51 *M. tuberculosis* strains were tested against the following final concentrations: 0.12, 0.25 and 1 μg/ml of isoniazid; 0.5, 1, and 2 μg/ml of rifampin; 0.5, 1, 1.5, 2, 4, 6 and 8 μg/ml of streptomycin and 1.25, 2.5, 5, 7.5 and 10 μg/ml of ethambutol. Bottles with the different drug concentrations and one drug-free bottle (control 1) were inoculated with 0.5 ml of a positive MB/BacT culture, a second drug-free bottle diluted 1:100 was also used as a control (control 2). All inocula were plated for cfu/ml counting. Criteria for susceptibility or resistance in bottles with the drug reading positive for growth in the MB/BacT System, were in reference to growth in both the control bottles. The proportions method on Middlebrook 7H10 solid medium, was used, according to the recommendations of the Centre for Disease Control (U.S.A.), as the reference method. Correlation between the method studied and the reference method was established for each drug concentration and the two control bottles. The time necessary for detecting resistant strains was evaluated for both of the controls.
Results: The best correlation between MB/BacT and the reference method was obtained with 0.25μg/ml of isoniazid, 1.2ml of rifampin and 2.5μg/ml of ethambutol, in these case the correlation obtained was 100%. The correlation for streptomycin was 95.2% using 1 μg/ml. The average time for detecting resistance was 4.6 days ranging from 2.5 to 10 days. Factors affecting the time necessary for detecting resistance were inoculum size and the period of time between pasteurization of a culture and the performance of the tests. The average time for detecting growth in the control 1 was 7.5 days and 6.6 days in the control 2.
Conclusion: The MB/BacT liquid media showed good correlation with the reference method. Further studies are needed.

Using mycobacteriophage D29 for rapid detection of drug resistance

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Heightened awareness of the problems of drug resistant infection has resulted in an increased demand for monitoring the drug susceptibility of *Mycobacterium tuberculosis* isolates.
Molecular methods and automated liquid culture methods offer more rapid screening but their high cost and the requirement for sophisticated technology limit their application in the poorly resourced laboratories found in many countries with a high burden of disease.
We demonstrate the use of mycobacteriophage D29 to detect susceptibility of mycobacteria to rifampicin, isoniazid, streptomycin and ethambutol. We demonstrate the inhibition of phage replication by rifampicin and streptomycin, where continued phage replication indicates resistance of the host bacteria to the drug, thus enabling overnight identification of drug resistant strains. We demonstrate detection of the bactericidal effect of isoniazid and ethambutol allowing rapid susceptibility testing following exposure of mycobacteria to these drugs.
The technology we are using is simple, inexpensive and does not require sophisticated equipment which should greatly facilitate its transfer to microbiology laboratories in less developed countries.
For screening of *Mycobacterium. tuberculosis* isolates for susceptibility to rifampicin we have developed a microplate format for convenient testing of large numbers of samples. Results from isolates cultured on solid media are obtained within 48 hours.

Rapid detection of rifampicin resistance in *Mycobacterium tuberculosis* by line probe assay

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The molecular mechanism of Rifampicin (RMP) resistance is well documented in the past several years: RMP-resistant strains of M. tuberculosis exhibit a genetic alteration, mainly a single nucleotide mutation, in the rpoB gene that encodes the b subunit of the RNA polymerase.
The INNO-LIPA Rif TB Kit was evaluated for rapide detection of RMP resistance in Mycobacterium tuberculosis clinical isolates: a target region (257 bp) from rpoB gene was amplified by Polymerase Chain Reaction. The Line Probe Assay, based on reverse hybridization principle, showed that RMP-resistance is due to a single nucleotide change in the codon 531 (12 strains), 526 (11 strains) and 516 (4 strains). In 10 RMP-sensitive strains the hybridization with the wild-type oligonucleotide probes confirmed the wild sequence of the target region. In contrast, tow multidrug resistant strains hybridized with the wild-type probes indicating that other mutations located out of the target region may be responsible for the rifampicin resistance.
The rapid detection of rifampicin resistance may contribute to management of MDR tuberculosis and limit the transmission of MDR strains.

Laboratory diagnosis of *M. xenopi* pulmonary infection–one isolated case in a non–immunocompromised female patient

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Human infections caused by *M. xenopi* have been found since 1960. *M. xenopi* is most frequently responsible for opportunistic pulmonary infections, clinicaly, radiologicaly and histologicaly similar to pulmonary tuberculosis. *M.xenopi* rarely produces non pulmonary lesions in patients who are not immunocompromised. An isolated case of a 30 year old female patient of *M. xenopi* pulmonary infection was detected in our laboratory. The isolation and identification of the ethologic agent was fundamental for TP exclusion. Microscopy, time and growth temperature, macroscopic and microscopic colony morfology and biochemical tests are the basis of the laboratory diagnosis. The aim of this study is to present and select the most important procedings to identify *M.xenopi* from other Mycobacteria of Runyon groups II and III, in order to make it as quickly as possible. Several culture media were used, i.e., Lowenstein-Jensen, Ogawa, Ordinary agar and Middlebook 7H10 agar, three different incubation temperatures (30º, 37º, 42ºC). Biochemical tests performed were: catalase activity (22º, 68ºC), catalase activity > 45mm foam, nitrate reductase and urease activity, tween 80 hidrolysis, iron up take, drug resistance to 1,0μg/ml INH, 10,0μg/ml INH, 5,0μg/ml EMB, 2,0 μg/ml Tb1, 10,0 μg/ml Tb1 and 5,0μg/ml PAS.
We concluded that bacilli morfology on microscopic observation, growth at 42°C temperature and X-colony morfology in 7H10 are fundamental for first aproach. Susceptibiliy to 1,0μg/ml INH, 10,0μg/ml INH distinguishes *M. xenopi* from other potential pathogenic non-photocromogenic species specialy *M. celtatum* and *M. avium* complex; 5,0μg/ml EMB plus tween 80 hidrolysis alows distinction from non pathogenic strains of Runyon groups II and III.

Radiometric broth dilutions method and e-test agar method for clarithromycin MICs of *M. avium* complex, *M. xenopi* and *M. scrofulaceum* clinical isolates

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Clarithromycin is an important drug in Mycobacteria Other Than Tuberculosis (MOTT) infections. Since there are no established methods for drug susceptibility testing of these organisms, broth dilutions minimal inhibitory concentrations (MIC) seems to be the option because MIC test on solid media with several drug concentrations was found not to be sensitive enough to make a distinction between isolates with different degrees of susceptibility and resistance. E-test agar MIC method apparently solved that problem. The aim of this study is to compare the MIC results obtained with radiometric broth dilutions method at two different pHs (6,8 and 7,4) with E-test agar system, in order to be able to implement one of the methods in routine clinical drug susceptibility testing for MOTT strains.

Reference strains of *M.avium* complex, *M.intracellulare* and *M. scrofulaceum* are tested and also clinical isolates of *M.avium* complex from HIV+ and HIV- patients, *M.xenopi* and *M. scrofulaceum* from pulmonary infections in non-immunocompromised patients.

Radiometric determination of MIC are made using Middlebrook 12B vials and Bactec 460TB apparatus (Becton Dickinson). All MICs are determined in parallel at two different pHs, i.e., pH6,8 and pH7,4. The concentrations tested are 0,05 - 0,1 - 0,2 - 0,4 - 0,8 - 1,6 ug/ml and 0,25 - 0,5 - 1,0 - 2,0 - 4,0 - 8,0 ug/ml. Clarithromycin was provided by Abbott laboratories. E-test agar MIC determinations uses 7H11 plates and clarithromycin strips were provided by the manufacturer (AB biodisk). All clinical isolates were identified in the laboratory by biochemical tests and DNA/RNA probes.

Because the study is not yet concluded, global results are not available ; discussion and conclusions will be later presented.

Use of Line Probe Assay (INNOLIPA) for rapid detection rifampicin resistence from clinical specimens

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AIM:Evaluation of a new technique (LINE PROBE ASSAY, LIPA MUREX) for easy and rapid detection of Rifampicin resistence (RMPr) of M.tuberculosis in clinical specimens.

MATERIALS AND METHODS: 50 clinical specimens (44 sputa and 6 bronchial aspirates) culture positive for M.tuberculosis were collected and aliquots of the decontaminated specimens were keept at -20°C.

The specimens were analyzed for RPM resistence by the Inno lipa RifTB test and the results were compared with susceptibility test to antituberculous drugs on L.J. and BACTEC TB test.

RESULTS:All samples hybridized to the M.tuberculosis complex specific probe and all produced Lipa interpretable results. 34 susceptible specimens produced a Lipa pattern of wild type not indicating any mutations of *rpoB* gene, according to L.J. and BACTEC drug sensitivity testing. Among 16 resistent specimens, 14 had mutated *rpoB* gene, in two samples the precise mutation could not be localized.

CONCLUSION:Lipa test is a fast method to detect both the presence of M.tuberculosis complex strain and its resistance to Rifampicin directly in clinical specimens.

Characterization of *rpoB* mutations in rifampin–resistant *Mycobacterium tuberculosis* clinical isolates from Greece

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There is a geographic distribution of *rpoB* gene mutations in *Mycobacterium tuberculosis* accounting for rifampin resistance. We studied seventeen rifampin-resistant clinical isolates from patients in Greece to identify rpoB mutations. We used automated sequence analysis of amplified PCR products and evaluated a commercial line probe assay kit (INNO-LiPA Rif. TB, Innogenetics). The overall concordance of the line probe assay kit with rifampin susceptibility testing was 94%. Three distinct *rpoB* mutations in codons Ser531, His526 and Asp516 were correctly identified with the kit, but mutations in external regions and insertions were detected only by automated DNA sequence analysis. The changes in codons Ser531 and His526 accounted for the majority of rifampin resistance, as previously described in other geographic areas. The results obtained with Random Amplification of Polymorphic DNA (RAPD) analysis of the tested isolates suggested that clonally related *M. tuberculosis* can have subclones bearing distinct mutant *rpoB* alleles.

We conclude that this line probe assay kit can be used for the rapid detection of rifampin resistance in *M. tuberculosis* before the availability of results with conventional methods and for epidemiological studies, but that negative results with this method do not rule out rifampin resistance.

Isolation of *Mycobacterium tuberculosis* by not radiometric system in a Universitary Hospital

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OBJETIVE:

In this work we reflect the incidence of isolation of mycobacteria on our environment by an automatic system (BACTEC 9000 TB) comparing with the conventional Lowenstein Jensen medium (LJ).

MATERIAL AND METHODS:

A total of 1228 clinical specimen were studied during sixteen months. All the specimens were decontaminated with sodium lauryl sulphate and 0,5 ml was inoculated in parallel in BACTEC MYCO/F (BACTEC 9000 TB) bottles and vials of Lowenstein Jensen (LJ). The samples were incubated at 37° during forty days.

RESULTS:

Mycobacteria were isolated in 173 samples (14,08%) by one of these systems. 167 (96,53%) was detected by BACTEC 9000 TB system and 140 (80,92%) by LJ. 143 mycobacteria grew in both medium (77,54%) , 33 (19,07%) strains were detected only by BACTEC 9000 TB and other 6 (3,46%) only by LJ.

The average time of mycobacterial growth in the BACTEC 9000 TB system was 344,38 h (14,35 days) and 648 h (27 days) for LJ. The technique of Ziehl Neelsen (ZN) were making in of 82,55% samples. The concordance between BACTEC 9000 TB and ZN was of 75,81% and between LJ and ZN was 97,24%.

CONCLUSIONS:

The sensitivity and reduction in the time of mycobacterial growth in the BACTEC 9000 TB system was notably better than LJ , even so there is a 3,46% of samples that only grow in LJ. Then its use in combination with BACTEC 9000 TB seems to be recommended to detect positive isolation. By the same way, the BACTEC 9000 TB system is more sensitive than ZN for the mycobacteria detection.

Bactec 9000 BM: one year of experience

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Objectives: To present our experience, evaluating the system Bactec 9000 MB for the isolation of Mycobacteria.
Material and methods: A total of 1889 clinical specimens were studied during one year (April 97 to April 98). Specimens processed for Mycobacteria cultures included respiratory specimens (sputum and bronchoscope specimens), gastric fluid, urine, pus, blood and body fluids (pleural fluid, ascites and cerebrospinal fluid).
From each specimen, a smear was prepared and was stained by the auramine fluorochrome method, excluding gastric fluid and blood specimens. Specimens were digested and decontaminated with N-acetyl-L-cysteine/NaOH and inoculated in bottles Bactec Myco/F and vials of Lowenstein-Jensen. The samples were incubated at 37º C. Blood specimens were cultured in bottles of Bactec Myco/F Lytic. Identification and susceptibility testing of isolated Mycobacteria were performed by the reference laboratory for Mycobacteria (Instituto Nacional Ricardo Jorge - Porto).
Results: From the total of 1889 specimens studied, 1662 (87,5%) were sterile, 124 (6,5%) were contaminated and 103 (5,9%) were positive. From these specimens, that corresponded to 72 different patients, we only evaluated a positive specimen per patient. From these isolates, 33 (45,8%) were recovered from respiratory specimens, 12 (16,7%) from pus, 10 (13,8%) from gastric fluid, 9 (12,5%) from sterile body fluids, 7 (9,7%) from urine and 1 (1,4%) from blood specimen. From the positive specimens, 25 (40%) were acid-fast smear-positive and 37 (60%) were acid-fast smear-negative. Detection times of Mycobacterial growth in Bactec were from 2 to 31 days (average 18,2) and from 8 to 75 days in Lowenstein-Jensen (average 30,9). Four isolates (5,5%) grew in both media at same time. In one sample, Mycobacteria were only isolated in the solid medium (1,4%). 92,8% isolates belonged to the M. tuberculosis complex and 5,3% were multidrug-resistant (Isoniazid and Rifampin).
Conclusions: This system is a rapid and sensitive method for isolation of Mycobacteria in a clinical lab, although it should be associated with a solid medium for a better recovery of such bacteria.

Comparison of two fully automated liquid culture systems for mycobacteria: bactec MGIT 960 and MB/Bact preliminary results

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520 specimens (except blood) from 279 hospitalised patients with pulmonary injuries suspected for tuberculosis were tested for mycobacterial growth in a routine TB-laboratory. After Nalc-NaOH pretreatment the specimens were cultured on two liquid media in the fully automated MGIT and MB/BacT systems (500ul. each) and on conventiane solid media Ogawa and Stonebrink for 8 weeks. Microscopy was performed by Acridine-orange fluorescent staining of the sediment. The aim of the study consisted in comparing the detection potency, the time needed for culture positivity, both in relation of mycobacteria of the tuberculosis complex group and of the „atypical" mycobacteria and the apparative handling in the routine laboratory.
Results: In Combination with conventional solid media both systems were similar in detection of mycobacteria (TB-complex and atypical mycobacteria). The detection time for TB-complex with the MB/BacT systems was 1 day longer than with the MGIT-system (17 vs 16days, averaged). For the atypical mycobacteria the difference was significant. The cultures (all atypical) became positive within 5.1 days in the MGIT and 10,1 days in the MB/BacT. For MAI-complex, the delay was 16 days in the MGIT and 31 days in the MB/BacT. The two automated systems were similar in the detection of TB-complex as atypical mycobacteria but differed in the culture time of atypical mycobacteria.

Evaluation of *FAST*Plaque TB for the rapid detection of *Mycobactrium tuberculosis*

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*FAST*PlaqueTB is a rapid assay capable of detecting *Mycobacterium tuberculosis* (MTB) in 48 hours from receipt of sample. An evaluation of the assay was conducted in South Africa which involved randomly selecting and interrogating a total of 240 samples (both pulomonary and extrapulmonary) for the presence of MTB using *FAST*PlaqueTB and routinely used laboratory methods of detection and confirmation (i.e. AFB smear microscopy and culture). The *FAST*PlaqueTB system has been configured to operate with decontaminated samples and harnesses the inherent sensitivity and specificity of mycobacteriophage. The assay allows for infection of MTB cells after which a selective virucidal agent is added to destroy the 'free' mycobacteriophage particles. Subsequent replication and release of progeny mycobacteriophage are detected by means of plaques upon a lawn of non-pathogenic, fast-growing cells.

Preliminary data analysis indicates that data generated using *FAST*PlaqueTB is comparable to 4-week Bactec (Becton Dickinson) data (38% and 37% positivity, respectively). *FAST*PlaqueTB is a novel, rapid, sensitive, specific and user-friendly MTB detection system.

Integrantig the MB BacT rapid culture system into routine diagnostic use

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Between May 1996 and June 1997 the Organon-Technika MB BacT non- radiometric liquid culture system was evaluated and compared to the Becton Dickinson Bactec 460 system at the PHLS MRU. Three different decontamination systems were used for respiratory samples. A trial comparing MB culture bottles with or without vancomycin supple- mentation was also undertaken. The three methods (quoting initial concentrations)were 23% trisodium phosphate (TRIP),2%sodium hydroxide(NaOH) with N-acetyl cysteine(NALC) and 4% NaOH-NALC. Positive isolation rates in the MB-BacT for all myco bacterial species were 9.3% (86 out of 927)with TRIP, 9.6% (9 out of 93)with 2% NaOH-NALC and 7% (13 out of 185) with 4% NaOH-NALC. Average times to detect all M. tuberculosis isolates using these decontaminat- ion methods were 18.5 days using TRIP, 10 days using 2%NaOH-NALC and 18 days using 4% NaOH-NALC. The overall contamination rates in the MB BacT were 14% with TRIP, 13% with 2%NaOH-NALC and 3% with 4%NaOH-NALC. After further analysis 4% NaOH-NALC is now used for decontamination of most respiratory specimens. Routine use of the MB BacT in parallel with solid egg media commenced in November 1997 replacing the Bactec 460 for all but blood/bone marrow specimens.

Clinical evaluation of MB/BACT and comparison to solid media for isolation of mycobacteria from clinical specimens

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The MB/BacT automated system (Organon-Teknika) is designed for the isolation of mycobacteria. In a closed system, it relies on a continuous colorimetric CO2 detection device by means of a solid state sensor at the base of each bottle. We compared the performance of MB-BacT system to solid media Löewenstein-Jensen (LJ) (Diagnostics Pasteur) for detection of mycobacteria. Non sterile specimens were digested and decontaminated by 4% NaOH procedure. The final sediment was shared out (i) on MB/BacT bottle (500 µl) and (ii) at least on 2 LJ slants (400µl). For contaminated specimens, MB/BacT Antibiotic Supplement + Vancomycin were added. MB/BacT bottles and LJ were incubated at 37°C respectively 6 and 12 weeks.

A total of 919 specimens (757 respiratory specimens, 113 body fluids, and 49 biopsies or other types) were cultured. Mycobacteria were recovered from 117 (12,7%) specimens : 66 *M. tuberculosis* (MTB), 5 *M. avium* complex, 31 *M. gordonae*, 3 *M. fortuitum*, 11 *M. xenopi*, and 3 *M. terrae*. In recovering MTB, 57 specimens corresponding to 24 patients were positive with MB BacT and 52 (21 patients) with LJ. The mean time to detection for smear positive or negative specimens was respectively 14,7±6 (5 to 32) and 21,4±5 (7 to 43) days by MB/BacT and 23,8±8 (17 to 66) and 29,7±10 (17 to 67) days by LJ.

The MB/BacT system offers a sensitive non-radiometric method for rapid detection of mycobacteria.

The isolation of non-tuberculous mycobacteria from a continuous automated liquid culture system (MB/BacT)

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The value of Continuous Automated Liquid Culture (CAMLiC) systems in the rapid isolation of Mycobacterium tuberculosis has been previously described. However, data on the impact of such systems on the isolation of non-tuberculous mycobacteria have been too few to draw accurate conclusions. We have now had three years experience in the use of the MB/BacT system in the routine laboratory diagnosis of mycobacterial infections. This presentation will give comparative data on the isolation of important non-tuberculous species. Isolation numbers and rates will be compared with results from standard egg-culture and conclusions presented on the value of automated liquid culture for these organisms.

Evaluation of two liquid culture-media (MGIT and MB-REDOX) in comparison with Bactec and Lowenstein–Jensen

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For the isolation of *Mycobacteria* from clinical specimens, the use of both liquid as well as solid media is recommended. The MB-REDOX™ (BIOTEST) and BBL® MGIT™ (DECTON-DICKINSON) are new, non-radiometric, rapid detection systems for the culture of *Mycobacteria* from clinical samples. Moreover, MB-REDOX is a colorimetric method and MGIT is a fluorometric system.

In order to evaluate MB-REDOX and MGIT, a comparison was made with two other methods currently in use: the classic Lowenstein-Jensen (L.J.) culture and the radiometric method BACTEC (DECTON-DICKINSON). 480 clinical respiratory samples - especially selected from patients suffering from pneumonic diseases - were studied. After the initial process, the samples were cultured in parallel in all four systems (methods), according to the manufacturers' instructions. Simultaneously for all these samples that were cultured there was a previous Ziehl-Neelsen stain. 25.6% of the samples give positive cultures in at least one of the methods used and 74.9% of the positive samples gave positive microscopic results. By L.J. method 87.8% of the positive samples give positive cultures, by BACTEC 94.3%, by MGIT 92.7% and by MB-REDOX 93.5%. The mean time of growth in L.J. was 21 days, in BACTEC 14 days, in MGIT and MB-REDOX 15 days.

In conclusion, we observed faster *Mycobacteria* growth using newer methods in contrast with L.J. However, MB-REDOX and MGIT gave swift and reliable results and it seems that they possess the advantages of *Mycobacteria* cultures in liquid media. Also, by comparison with MGIT, MB-REDOX allows the easy, immediate macroscopic reading of the results, without using additional facilities.

Diagnosis of mycobacterial infection using a bacteriophage assay

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It has been estimated that 95% of tuberculosis cases occur in the less developed countries. There is an urgent need for more sensitive, rapid diagnostic tests that are appropriate and affordable for use in low-income countries.

Mycobacteriophages are viruses that infect and replicate in mycobacteria. Their potential for use as tools for diagnosis and drug susceptibility testing is currently under investigation. Luciferase reporter phage have been produced which allow rapid assessment of viable bacteria. Alternatively a highly sensitive 'low-tech' method has been developed in which phage replication is detected by the production of visable plaques in a lawn of fast growing mycobacteria. The method relies on the ability of ferrous compounds to differentially inactivate phages in liquid broth while not affecting ongoing phage replication within mycobacteria. Using 10mM ferrous ammonium sulphate we have demonstrated detection of less than 10 cfu of *Mycobacterium smegmatis*. The assay can be used detect viable *Mycobacterium tuberculosis* in clinical specimens of sputum, providing a diagnostic result within 48 hours of receipt of the specimen.

This simple low-cost technology may prove appropriate for those laboratories with limited resources for investment in the improvement of TB diagnosis.

Evaluation of an automated system (MB/BacT®) for isolation of *Mycobacterium spp*

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The study purpose was to evaluate the MB/BacT automated system against Löwenstein Jensen solid media for the isolation of Mycobacterium species. 4,017 specimens were processed from 15/10/96 to 21/4/98. For digestion and decontamination NALC-NaOH was used. Auramine smear was performed and both culture media were inoculated after digestion-decontamination. Inoculation on MB/BacT bottles was performed according to the manufacturer's instructions, apart from a modificacarion in MAS antibiotic supplement with the addition of vancomycin at 1mcg/ml as final concentration. Both media were incubated for 40 days at 37ºC.

In total 253 (6,3%) mycobacteria species were isolated: 221 *M. tuberculosis*,19 *M. avium*, 6 *M. gordonae*, 1 *M. Kansasii*, 2 *M. chelonae* and 1 *M. fortuitum*. 240 (5,9%) *Mycobacterium spp.* were isolated in MB/BacT and 195 (4,9%) were isolated in Löwenstein Jensen. 58 (1,4%) out of 253 grew only in MB/BacT and 13 (0,3%) out of 253 grew only in Löwenstein Jensen.

Sensitivity in MB/BacT was 94,9% compared to 77,1% in Lowenstein Jensen, this difference was statically significant (p<0,001).

Contamination rate was 2,6% in MB/BacT and 1,4% in Löwenstein Jensen. The average time to detection of *Mycobacterium spp.* was 16,6 days in MB/BacT and 21,9 in Löwenstein Jensen.

We believe that MB/BacT is a good system for rapid and reliable detection of *Mycobacterium spp.*

Evaluation of recovery and time detection of *M. tuberculosis* in the MB/Bact culture system compared with micobacterial growth indicator tube (MGIT), Bactec 460 and Lowenstein – – Jensen medium

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OBJETIVE
Mycobacterial recovery and time detection in the MB/Bact system (MB)(Organon Teknika) were compared with MGIT (Becton Dickinson), radiometric BACTEC 460 TB system (12B)(Becton Dickinson) and Lowenstein-Jensen (LJ) solid media (BioMerieux).
MATERIAL AND METHODS
A total of 300 processed specimens were inoculated into all media in parallel. Specimens included 231 from pulmonary sites and 69 from extrapulmonary sites. Respiratory specimens and urine samples were decontaminated by the Isoquet and Tison method. All vials were inoculated with 500 µl of samples and LJ with 200 µl each one. The vials were incubated at 37ºC for 8 weeks.
RESULTS
120 specimens (24%) were positive for *M.tuberculosis*. MB detected 98 (81,6%), MGIT 91 (75%), 12B 101(84,2%) and LJ 101 (84,2%). The average number days for MB to detection single mycobacterial isolates was 17,9 days for MB, 22,4 days for MGIT, 14,5 days for 12B and 20,5 days for LJ. The contamination rates were 5,2 % for MB, 2,5 % for MGIT, 3 % for 12B and 7,2 % for LJ.
CONCLUSIONS
The MB/Bact culture system is a most valuable rapid culture system for the isolation of *M.tuberculosis* from clinical samples. To achieve minimal contam rates it should be used in parallel with conventional media.

Comparison of the BACTEC™ MGIT™ 960 systems with solid media for recovery of mycobacteria from clinical specimens

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Objectives: This study was performed to evaluate the performance of the BACTEC™ MGIT™ 960 System in comparison to solid media (Lowenstein Jensen with PACT (LJP) and Coletsos), for the recovery and time to detection of mycobacteria from human clinical specimens.
Material and Methods: A total of 1253 specimens were processed. The specimens were decontaminated according to the standard CDC NALC NaOH method, using the commercial MycoPrep kit (Becton Dickinson). The specimens included : 1100 sputa, 90 urines, 2 bronchial washings, 4 gastric fluids, 3 pleural fluids, 1 pericardial fluid, 1 peritoneal fluid, 8 CSF, 3 exudates, 12 bone marrows, 13 tissues, 11 lymphnadeno and 5 other specimens.
Results: A total of 234 (18,7 %) mycobacterial isolates were detected : 224 (95,7%) in MGIT, 178 (76.1%) in LJP and 182 (77.8%) in Coletsos. 48.8% of the positive cultures were smear positive.
The mean times to detection in MGIT, LJP and Coletsos were 9.4, 24.6 and 23.2 days for *M. tuberculosis*, 19.1, 51.0 and 25.6 days for *M. bovis*, and 5.2, 20.7 and 17.6 days for *M. avium*, respectively.
The contamination rates in MGIT, LJP and Coletsos were 6.5%, 8.5% and 14.1%, respectively. 19 (1.5%) of the specimens were contaminated in all media.
Conclusions: The non-radiometric BACTEC™ MGIT™ 960 is a compact system for growth and detection of mycobacteria from clinical specimens. Compared to solid media sensitivity and time to detection are significantly better.

Comparison of COBAS® AMPLICOR MTB automated system and smear microscopy and culture in routine TB diagnostics

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COBAS® AMPLICOR is first fully automated system for routine PCR diagnostics. It combines amplification and detection by hybridisation probe, with possibility of connecting sample preparation module in the future. *M. tuberculosis* complex was among the first three tests adapted for COBAS AMPLICOR (see our pilot study, Pacis 1996).
In our recent study, results obtained during our 26 month experience in routine use of COBAS AMPLICOR automated system in parallel manner with other methods of routine diagnostics of TB are summarized.
900 hundred sputum samples and 200 BALs obtained from pulmonological clinic patients were examined. There was no difference in final diagnosis between CA and Culture in the group of smear microscopy highly positive samples. In samples with race rods in ZNI and BALs discrepant analysis occured. These samples were additionally cultured also in MB/Bact system in order to solve the discrepancies and further identified by Gen-Probe Accuprobes. The results were also consulted byclinicians. Results of different test were afterwards compared with confirmed infection. COBAS AMPLICOR is really suitable detection system for MTB complex, equiped by system of sofisticated controls to prevent both the false positive and false negative results.

Multicenter evaluation of the BACTEC™ MGIT™ 960 system compared to the BACTEC™ 460 TB system and solid media for recovery of mycobacteria

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In a multicenter study involving six centers for mycobacteria, the rate of recovery of acid-fast bacilli (AFB) and the mean time to their detection from clinical specimens was determined by using the automated BACTEC MGIT 960 system (MGIT 960). These parameters were compared to those assessed by the radiometric BACTEC 460TB system (BACTEC) and by cultivation on solid media. MGIT 960 contains a modified Middlebrook 7H9 broth in conjunction with a fluorescence quenching-based oxygen sensor. The system reads the tubes for fluorescence hourly and was developed to simultaneously monitor 960 tubes.

Clinical specimens (n= 3,330) from both pulmonary and non-pulmonary origins were pretreated with NALC-NaOH. A total of 362 mycobacterial isolates were detected (*M.tuberculosis* complex, n= 132; nontuberculous mycobacteria, n= 230). When using the current „gold standard" for culture (a combination of liquid and solid media), MGIT 960 plus solid media detected 335 (93%) of the isolates, whereas BACTEC plus solid media recovered 308 (85%) of all AFB. The mean time to detection was 11.8 days with MGIT 960, 12.5 days with BACTEC, and 24.1 days with solid media. The breakthrough contamination rate for MGIT 960 was 8.1%, and for BACTEC 4.9%. The false positive rate (a positive instrument reading but no AFB or other microorganisms are observed) was 0.8% for MGIT 960 (BACTEC: 0.4%).

In conclusion, MGIT 960 is an automated, accurate, nonradiometric, noninvasive alternative to BACTEC for rapid recovery of AFB, which can be used also for a high-volume mycobacteria laboratory.

Evaluation of the MB/BacT automated system for the detection of mycobacteria

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The MB/BacT (Organon Teknika), a rapid automated system for the detection of mycobacteria was evaluated on behalf of the UK Medical Devices Agency. A total of 476 samples were tested. These comprised mainly sputum (62%) from patients with suspected tuberculosis. Samples were digested and decontaminated for 15 minutes using the NALC - NaOH method; the final concentration of NaOH was 2%. The specimens were tested using the MB/BacT, BACTEC 460TB (Becton Dickinson) and solid media.

From the 476 sets, acid-fast bacilli were detected and seen in 54 samples from at least one of the systems. Acid fast bacilli were detected in 53 (98%) of the MB/BacT bottles, 47 (87%) of BACTEC bottles and in 47 cases (87%) using solid media. In three samples, contamination prevented isolation and identification of the acid fast bacilli (MB/BacT 2 samples and BACTEC 1 sample) and there were therefore 51 isolates. All fifty-one (100%) were isolated by the MB/BacT, 46 (90%) by the BACTEC and 47 (92%) using solid media.

The mean detection intervals for all mycobacteria for each system were MB/BacT 15 days, BACTEC 15 days and solid media 25 days.

The contamination rates were MB/BacT 8.4%, BACTEC 2.3% and solid media 5.5%.

In conclusion, the MB/BacT had a superior detection rate than the BACTEC and solid media despite having a higher contamination rate. The automated systems were also more rapid in detecting mycobacteria.

Evaluation of the BACTEC™ MGIT™ 960 system for growth and detection of mycobacteria in human clinical samples

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This study was performed to evaluate the performance of the BACTEC™ MGIT™ 960 in comparison to the BACTEC™ 460TB and solid media (Loewenstein lenses, Middlebrook 7H11 and 7H11S) for recovery and time to detection of mycobacteria from human clinical specimens. The BACTEC™ MGIT™ 960 uses MGIT tubes filled with 7 ml of a modified Middlebrook 7H9 broth containing an O_2 sensitive fluorescent sensor. O_2 consumption by mycobacteria allows the fluorescence to be detected. Liquid media were incubated for 6 weeks and solid media for 8 weeks.

697 specimens were processed and decontaminated according to the standard CDC NALC/NaOH method, using the commercial Mycoprep kit. The specimens included 455 respiratory (sputa, bronchial washings) and 242 non respiratory (body fluids, urines, pus and other).

A total of 110 (15.8 %) mycobacterial isolates were detected : 108 (98%) were detected with the fluorescent BACTEC™ MGIT™ 960, 107 (97%) with the radiometric BACTEC™ 460TB and 103 (94%) with solid media. These 110 mycobacterial isolates originated from 91 smear positive and 19 smear negative.

The mean times in detection were 5.7 days for smear positive specimens and 11.5 days for smear negative specimens with the BACTEC™ MGIT™ 960; 8.2 and 12.9 days respectively with the BACTEC™ 460TB; 21.3 and 29.1 days respectively with solid media. For all isolates the average detection times were 6.7, 7.4 and 22.5 days respectively.

The non-radiometric BACTEC™ MGIT™ 960 is a rapid, sensitive and less labour intensive detection system. It is efficient for the recovery of mycobacteria from both respiratory and non respiratory specimens.

Evaluation of a rapid immunochromatographic test for the diagnosis of active tuberculosis in a low–prevalence population

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We evaluated a commercially available immunochromatographic membrane-based assay (ICT Tuberculosis, ICT Diagnostics, Brookvale, Australia) for the detection of specific IgG antibodies to 5 antigens of *Mycobacterium tuberculosis* in patients with active tuberculosis in a low-prevalence population in Belgium.

Stored frozen sera from 45 patients with culture-confirmed active (pulmonary or extra-pulmonary) tuberculosis were tested along with 36 sera from control patients with respiratory diseases other than tuberculosis and 47 healthy controls.

The sensitivity of the test for active tuberculosis was 42 % (19 positives out of 45 patients); its specificity in the group of patients with other lung diseases was 92 % (3/36 false positives) and in the group of healthy controls 100 %. The sensitivity of the immunochromatographic test was equal to that of auramine staining, but different subsets of tuberculosis patients were detected by the two tests.

The suboptimal sensitivity of this immunochromatographic test implies that it cannot be a replacement for the diagnosis of tuberculosis by other microbiological methods along with clinical and radiological data. However, because the test is rapid and easy to perform, has a relatively high specificity, and detects some cases that are missed by auramine staining, it could be a useful adjunct test in the rapid identification of some patients suffering from tuberculosis.

Usefulness of the Accuprobe *Mycobacterium tuberculosis* complex culture identification test (Genprobe, San Diego, USA)

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The results of 1922 Accuprobe *M. tuberculosis* complex culture identification tests were evaluated. In total 98.7% of the tests gave clear negative (<1500 RLU), or positive results (>30,000 RLU). Of the 25 (1.3%) tests with doubtful results, only 6 (0.3%) remained in the 'grey zone' between 1,500 and 30,000 RLU after repeated testing using a higher cell number. These 6 isolates appeared to be contaminated with non-mycobacteria, or non-*M. tuberculosis* complex bacteria. All but one of the strains with positive Accuprobe test results contained the *M. tuberculosis* complex-specific insertion sequence *IS6110*, as proven in restriction fragment length polymorphism typing. The exceptional isolate without *IS6110* DNA was identified as *M. tuberculosis* on basis of other genetic and phenotypic criteria[1]. We conclude that (i) the Accuprobe test for the identification of *M. tuberculosis* complex bacteria has a specificity of nearly 100%, and (ii) the low percentage of doubtful results is reduced significantly after repetition with a higher cell number.

[1]Van Soolingen, D., P.E.W. de Haas, P.W.M. Hermans, P.M.A. Groenen, and J.D.A. van Embden. 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. J. Clin. Microbiol. 31:1987-1995.

Clinical evaluation of the amplified *Mycobacterium tuberculosis* direct test (AMTDT)

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When analyzing a new test, the performance data such as sensitivity and specificity are compared to the current „golden standard", which, in the case of tuberculosis diagnostics, is the cultivation of Mycobacterium tuberculosis strains in or on specific culture media.

During a time span of 39 months, 3353 clinical samples were examined using the first generation Amplified Mycobacterium Tuberculosis Direct Test (AMTDT). 70.9% of the samples were from the respiratory tract, 17.7% from the urogenital tract, and the rest were various other types of samples.

When the results of the AMTDT were correlated with not only the culture results, but also the patient history as well as the clinical and therapeutic information, the AMTDT results changed as follows:

Sensitivity:	from 79.8% to 88.2%
Specificity:	from 93.4% to 94.6%
False negative results:	from 20.2% to 11.8%
False positive results:	from 6.6% to 5.4%

In order to minimize the number of false negative AMTDT results, at least three consecutive samples should be analyzed from each patient suspected of having tuberculosis. When interpreting a positive AMTDT result, the patient history as well as the clinical and therapeutic aspects must be taken into account.

Bacteriological diagnosis of tuberculosis meningitis using the BACTEC 460TB system

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Tuberculous meningitis is a rare, severe and often fatal, form of the disease related to the dissemination of the *Mycobacterium tuberculosis* infection. A fast laboratory diagnosis is mandatory as isolation of the agent is essential for susceptibility testing. Since 1995, we received 484 cerebrospinal fluid (CSF) from 426 patients with clinical suspicion of tuberculosis with meningial involvement. The samples were collected from the several hospitals in Lisbon. All the samples were decontaminated by the N-acetil-L-cystein technique and inoculated in 13A or 12B BACTEC medium bottles (Becton Dickinson Inc USA) supplemented with PANTA (antibiotic mixture) and incubated at 37ºC for eight weeks. The average recovery time for positive cultures was three weeks. All positive cultures were identified by the p-Nitro-acetilamino-β-hidroxy-propiophenol (NAP) test and confirmatory biochemical tests. Susceptibility testing was performed according the BACTEC 460TB procedure manual for the following antituberculous drugs: Streptomycin, Isoniazid, Rifampin and Ethambutol. The results are depicted bellow:

SAMPLES (n=484)	FREQUENCY
POSITIVE	4,6% (22/484)
NEGATIVE	93,4% (452/484)
CONTAMINATED	2,0% (10/484)

All isolates were identified as *M. tuberculosis* (MTB) and the susceptibility testing gave the following results:

MTB STRAINS (n=22)	FREQUENCY
SUSCEPTIBLE	50,0% (11/22)
RESISTANT TO ONE DRUG	4,5% (1/22)
MULTIDRUG RESISTENT	45,5% (10/22)

We concluded that this laboratory procedure was appropriated for the rapid isolation, identification and drug susceptibility characterisation for *M. tuberculosis* in tuberculous meningitis, mainly due to the high % of MDR strains.

Detection of *Mycobacterium tuberculosis* in clinical samples by DIG-PCR Elisa system

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In Poland tuberculosis is the most important epidemiological and health problem due to infectious diseases. Tuberculosis has been the reason of nearly 50% of deaths caused by infectious diseases. The presence of *M. tuberculosis* in patient specimens is required for the definitive diagnosis of tuberculosis. The most rapid method of identifying *M. tuberculosis* to the species level in clinical samples is the PCR. The aim of our work was to establish the DIG-PCR ELISA system for detection and specific indentification of *M. tuberculosis* in clinical samples and evaluation of utility of this system for diagnosis of tuberculosis. This system allows the convenient and specific detection of PCR products which are labelled with digeoxygenin (DIG) during the amplification process. To detect *M. tuberculosis* directly in clinical samples we used the insertion elements: (i) IS6110 and (ii) IS990. One hundred clinical samples (sputum, bronchial wash, urine) obtained from patients during 1997-1998 were tested by DIG-PCR ELISA system. Our results were verified by conventional diagnostic methods (culture growth, biochemical tests and BACTEC.

This test may be helpful in the early and specific diagnosis of tuberculosis.

Isolation of a Specific DNA fragment and Development of a PCR–based method for the detection of *Mycobacterium genavense*

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The rise in the incidence of *Mycobacterium genavense* infections has made identification ever more important for diagnosis and treatment. The isolation and identification of *M. genavense* are made difficult by the lack of cultivation on solid media and by the limited multiplication of the organism in BACTEC liquid media. Thus, amplification by PCR or similar techniques represents the only possibility of detecting and identifying *M. genavense* from tissue specimens. In order to set up a simple and species-specific method based on the use of PCR and a non-radioactive hybridization technique, we decided to search for and clone a specific DNA fragment from this bacterial species. The 1734 bp fragment isolated in the present study was found to be highly specific for *M. genavense* strains. A species-specific pair of primers (MG22 and MG23) and two oligonucleotide probes (MG18 and MG19) were selected. They were successfully used to amplify and detect a 155 bp DNA fragment when the 13 strains of *M. genavense*, available for the present study, and isolated from clinical specimens or from birds were analyzed. Conversely, these primers and probes did not hybridize with DNA from any of the 20 other mycobacterial species tested. It is noteworthy that the chosen primers and probes do not hybridize with DNA from *M. simiae*, a mycobacterial species which is closely related to *M. genavense*. Other PCR assays have been described for the characterization of *M. genavense*. However, the present PCR technique is the first using species-specific primers for this mycobacterial species. This PCR technique followed by a non-radioactive hybridization technique on microplates is able to distinguish *M. genavense* from other mycobacteria in one step, without sequencing or restriction analysis.
On the basis of the Southern blot hybridization, PCR and sandwich hybridization results, we can conclude that the isolated 1.7 kbp sequence was specific for the *M. genavense* chromosome. The method developed here for *M. genavense* identification uses simple methodology and currently available reagents. Furthermore it can be easily automated.

Rapid diagnosis of tuberculosis by using Roche Amplicor *Mycobacterium tuberculosis* PCR test

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A total of 332 respiratory and 225 nonrespiratory specimens obtained from 477 patients were tested for detection of *Mycobacterium tuberculosis* complex (MTB) by a commercial polymerase chain reaction (PCR) kit (Amplicor, Roche Diagnostics) and the results compared with those of microscopy and culture (Coletsos and MGIT media). Respiratory and nonrespiratory specimens were analyzed separately. Of the respiratory specimens, 53 were positive for MTB both in the PCR and in culture, 21 were positive in the PCR but negative in culture, and 12 were positive in culture but negative in the PCR. Eighteen cultures were positive for mycobacteria other than MTB; 36 specimens (34 patients) gave a positive result in the PCR test.
Resolution of discrepant results was performed by analysis of patient's clinical data. For respiratory specimens the sensitivity of PCR test was 91.4%, the specificity 98.0%, the PPV 93.7% and the NPV 97.2%. For nonrespiratory specimens, the sensitivity was 85.9%, whereas the specificity was 94.4%. The PPV was 74.3% and the NPV 97.4%. On 238 specimens, 14 (5.9%) were inhibited and only 4 (1.7%) were always inhibited when a second diluted aliquot was tested. Moreover, the routine use of internal control procedures for monitoring inhibitory specimens increased the sensitivity. Potentially, all tuberculosis patients with smear-positive specimens can be immediately confirmed as being infected with MTB, leading to improved clinical management. A PCR-negative result for a single specimen should be considered very carefully and in order to obtain a high degree of sensitivity it should be necessary to test more than one specimen.

Amplified *Mycobacterium tuberculosis* test for the automated VIDAS instrument

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Objective: To develop an automated amplified *M. tuberculosis* (M.tb) assay pairing Gen-Probe's isothermal Transcription-Mediated Amplification (TMA) and Vitek's automated VIDAS immunoassay detection instrument. Novel enhancements include: single dose amplification reagents such as a pelletized enzyme reagent, enclosed strip format, and a co-amplified internal control to monitor specimen inhibition as well as reagent or operator error.
Method: Amplification of a respiratory specimen's rRNA occurs during the TMA reaction. The amplified product is then hybridized to a specific probe during detection on the VIDAS instrument.
Results: The amplified method showed complete specificity for members of the M.tb complex and did not cross react with a panel of 32 species of mycobacteria other than M.tb or 11 related genera. The sensitivity of the system is less than one M.tb cell per test. Four hundred sixty-three clinical specimens were tested and the results compared to culture. Sixty-one out of 62 M.tb culture positive specimens were also positive by the VIDAS amplified method, including 10 specimens that were smear negative for acid-fast bacilli (98.4% sensitivity). There was no cross reaction with 47 specimens identified by culture to contain mycobacteria other than M.tb. Three hundred seventy-four out of 376 specimens were negative for M.tb by both methods (98.9% specificity). The internal control successfully monitored specimen inhibition or other amplification or detection failures.
Conclusion: The alliance of probe diagnostic technology with the existing VIDAS automated instrument functioning in clinical laboratories worldwide will allow for rapid, accurate and cost-effective identification of *M. tuberculosis* in respiratory specimens.

Rapid detection of *M. tuberculosis* from cerebrospinal fluid using the Gen-Prob amplified MTD2 test

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The direct amplification tests (DAT) save time to detect *M. tuberculosis* complex (MTBC), not only from pulmonary, but also from extrapulmonary specimens. A second generation DAT, Amplified Mycobacterium Tuberculosis Direct 2 (MTD2) Test (Gen-Probe) has been developed as an adjunct to the acid-fast smear and culture for detection of MTBC from digested and decontaminated respiratory specimens. Unlike the MTD1 test that requires 50 µl of sediment, the MTD2 uses 450 µl. The time to detection of MTD2 was reduced from 4 hours to 2 hours. The test is targeted for respiratory specimens, but its versatility expands to extrapulmonary specimens.
This investigation demonstrates the successful use of the MTD2 in detecting MTBC from cerebrospinal fluids (CSF) that were obtained from patients who presented at our medical center in 1996 and 1997. From a total of 810 CSF specimens processed in 1996, 5 were positive for mycobacteria. Four specimens (2 patients) grew MTBC and a single specimen grew M. avium complex. In 1997,, 579 CSF specimens were received and one grew MTBC. The CSF specimens in all culture positive cases demonstrated predominantly lymphocytic pleocytosis with elevated total protein of > 45 mg/dl and decreased glucose of <40 mg/dl. Acid-fast smears of the specimens were negative and the cultures took 3-4 weeks to grow. These results indicate that : 1) CSF specimens are being inappropriately ordered (0.17% positive in 1997 and 0.5% positive in 1996) and 2) the MTD2 test results, which were available within one day of specimen collection, saved time to diagnosis considerably. Since tuberculous and nontuberculous meningitis are rare, clinical parameters must be used to indicate a high suspicion for disease.

Development of a quantitative diagnostic procedure for *Mycobacterium tuberculosis* infection

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We have developed a strictly quantitative technique allowing the precise determination of the mycobacterial load in clinical samples. This technique is based on the competitive PCR procedure, consisting in the amplification of the sample DNA with a specific competitor DNA sharing the same primer recognition sites. The target region is a 223 bp segment of the insertion element IS6110, specific for the MTB complex. Competitor DNA was constructed by insertion of a 20 bp fragment in the middle of the target sequence, in order to allow resolution by gel electrophoresis. The competitive PCR assay consisted in the coamplification of target DNA samples with scalar amounts of competitor DNA. The sensitivity of the procedure allows the specific detection of about ten molecules of target MTB DNA.

The suitability of quantitative PCR for monitoring antimycobacterial therapy was assessed in a preliminary study. To this purpose, specimens were collected from each patient and submitted to PCR on a weekly basis for the early therapy period. Quantitative analysis of recently hospitalized patients revealed the presence of more than 10^6 molecular targets per ml of tested sputum. This number decreased rapidly (by two orders of magnitude) within the first month in patients responsive to therapy.

This work was supported by the Istituto Superiore di Sanità, National Project on Tuberculosis

Clinical trials results using the enhanced amplified *Mycobacterium tuberculosis* direct (MTD) test

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Objectives: To determine the performance of the second generation MTD test by comparing the results of the test with patient diagnosis.
Methods: A simplified version of the MTD test, with fewer steps and a shorter amplification time was used to test respiratory sediments at 7 clinical trial sites, including 6 in the U.S. and one in Switzerland. Patients suspected of having tuberculosis and not receiving therapy were enrolled in the study. From 1-6 samples per patient were tested. Results of the MTD test were compared with the site physician's diagnosis. According to established criteria, cases which were less clear-cut were judged by a panel of physicians expert in the field of tuberculosis.
Results: A total of 337 patients contributed 831 specimens. The sensitivity, specificity, PPV and NPV of MTD relative to the patient diagnosis were 84.5%, 97.0%, 88.2% and 95.9%, respectively. The sensitivity, specificity, PPV and NPV of BACTEC relative to the patient diagnosis were 83.1%, 99.6%, 98.3% and 95.7%, respectively. The MTD results were available in one day. Culture results were available on average from 11 - 21 days.
Conclusions: These data underscore that the MTD test performs comparably with culture for detection of tuberculosis in patients who are suspected of having tuberculosis. However, the MTD results are available much more rapidly and can provide very useful information to the physician.

Utility of Polymerase Chain Reaction for the diagnosis of pulmonary tuberculosis in a rural african laboratory

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There has been a dramatic rise in the incidence of smear-negative tuberculosis in sub-Saharan Africa coincident with the HIV pandemic. During 1995 67% (19,319) of new notifications of pulmonary tuberculosis in Zambia did not have a positive smear result. We have been investigating the role PCR could play in diagnosis of pulmonary TB in a rural laboratory currently performing direct ZN smears where facilities to culture *M. tuberculosis* are not available.

During 1997 the laboratory performed on average 16.8 smears per working day of which 2950 (15.5/day) were from suspect cases. The numbers of specimens examined per suspect ranged from 1 to 7, with an average of 2.4 and a median of 3. Primary smears identified 148 (12.2%) of suspects as positive while examination of the second smear identified an additional 20 (1.6%) and the third specimen a further 7 (0.6%) cases. Microscopy of all subsequent specimens failed to identify any additional positive cases. In a pilot study using an 'in-house' PCR to test initial sputum specimens 6 (5.4%) were found positive by both PCR and smear; 3 (2.7%) were PCR positive/smear negative but subsequent smears from the suspect proved positive and 23 (20.5%) were PCR positive/smear negative.

Our analysis indicates that PCR of a single sputum specimen is more effective than repeated smears for improving the detection of pulmonary tuberculosis.

Co–operation of clinician and laboratory in interpreting results of examination by *Mycobacterium tuberculosis* direct test

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In 1995, the Department for Mycobacteria diagnostics of the Regional Institute of Hygiene (KHS) in Ostrava performed examination of 1009 samples simultaneously by culture, MTD test and, except for urine samples and LV, also microscopically. In 50 patients with negative results obtained by classical methods, a positive MTD test was found. 23 of these patients were finally diagnosed as having pulmonary tuberculosis and 4 extrapulmonary tuberculosis, in both cases unconfirmed by bacteriological investigation. Another 4 patients have been diagnosed as A 15.3 according to the International Classification of Diseases - TB confirmed by other methods - in this case by MTD test. In 15 patients with positive MTD test, the attending physician has ruled out TB aetiology of the disease.

All patients remained further followed by the Specialised agency for tuberculosis and respiratory diseases, which has been asked to follow and evaluate the condition of the patient's health for 3 years.

Clinically, the original diagnosis of tuberculosis has been confirmed in all cases, in one of the patients with "false-positive results" the original diagnosis of pleuropneumonia has been reassessed to tuberculosis secondary to pneumoconiosis.

Comparative study of PCR and LCR methods in the diagnosis of tuberculosis

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Objectives: The performance of two DNA amplification techniques, Roche Cobas Amplicor polymerase chain reaction (PCR) and Abbott ligase chain reaction (LCR), was evaluated for tuberculosis diagnosis.
Materials and Methods: A total of 72 specimens from 45 patients were studied (14 HIV positive), all of them collected from February 1997 to June 1997: 65 respiratory samples and 7 nonrespiratory samples. Decontamination was done according to the NaOH method. Microscopy was done both by auramine stain and Ziehl-Neelsen stain and then all samples were assayed by two solid media and radiometric BACTEC liquid method. The PCR and LCR assays were run according to the instructions of the manufacturer. The results were compared to culture and microscopy and discordant results were resolved by patient history according to the guidelines and classification of tuberculosis from the American Thoracic Society (1990).
Results: The overall sensitivity and specificity as compared to culture for *M. tuberculosis* were 43.7 and 96.4% for stain, 68.7 and 69.6% for PCR and 68.7 and 85.7% for LCR, respectively. Samples (20) from 9 cases not treated (class 3) were found positive by stain 3, by culture 12, by PCR 13 and by LCR 8. Specimens (15) from 6 cases treated (class 3) were found positive by stain 6, by culture 5, by PCR 8 and by LCR 11. On an other hand, 4 samples from 3 cases with history of tuberculosis (class 4) all of them were negative from stain, culture and LCR negative, but 3 were PCR positive. Finally, 1 specimen from a class 2 patient was only PCR positive.
Conclusion: More than one sample from each patient should be tested. The evaluation of different rapid amplification techniques should consider the broad host-parasite relationships as well as the heterogeneity of tuberculous patients.

Long–term evaluation study on Gen-Probe MTD test

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Gen-Probe Amplified Mycobacterium tuberculosis Direct test (MTD) is connercially available assay for detection of M.tuberculosis complex 3074 different samples were examined in a parallel manner (2445 sputa from 1912 patients, 629 bronchoalveolar lavages from 443 patients and 154 other samples)
The study was made from November 1995 till December 1997 and covered Prague and Central Bohemia region (2 million inhabitants). All samples were decontaminated using NaCl-NAOH and examined by MTD test, Smear microscopy(auramine fluorescence) and Culture (Loewenstein-Jensen's media).
520 sputum and 109 BAL samples obtained from patients with confirmed pulmonary TB were positive in all tests(MTD, SMI, Culture), 71 additional sputum and 19 BAL samples were positive in MTD test only. Out of this group, 43 patients were subsequently confirmed clinically as positive. 20 patients were in long-term anti-TB treatment, 5 patient suppose coincidence of TB and tumor, 3 patients suffered borreliosis (high-antibody titres). 56 sputa from patients with clinical symptoms were positive in SMI and Culture only. All these discrepant samples were subsequently resolved. Gen-Probe MTD test showed fully suitable for routine MTB testing.

Evaluation of PCR amplicor *Mycobacterium avium/intracellulare* kit for the diagnosis of MAC infections

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The symptoms of *M. avium/intracellulare* complex (MAI) disseminated infections are not specific, the detection and the identification of the bacteria requiring 10 to 20 days minimum are necessary to confirm the MAI infection. The aim of this study was to evaluate the Amplicor kit (Roche Diagnostics) using PCR and specific hybridation for direct detection of *M. avium* (MAC), *M. intracellulare* (MIC) and *M. tuberculosis* (MTB) and with an internal control to check the absence of substances inhibiting PCR.
This evaluation was done in 1996-1997 on 123 samples (48 blood samples from VIH + patients, 46 respiratory samples and 29 nonrespiratory specimens). The PCR results were compared with those obtained by culture (Septi-Check AFB (Becton Dickinson)) for blood samples collected on Isolator tube (Oxoid) and, Coletsos and MGIT (Becton Dickinson) media for other samples.
The detection limit of Amplicor kit allowed to detect up to 10 bacilli/ml. No amplification product has been obtained from 13 DNA of bacteria supposed to be present in the bronchial tractus. On 48 blood samples, 2 were culture and PCR positive, 1 was positive only in the PCR and 8 inhibitory specimens. On the 75 other samples, 53 were negative, 1 biopsy was MAC positive, 6 respiratory samples were MIC positive, 10 samples were MTB +, 4 inhibitory specimens and for one biopsy the result was doubtful. All the PCR results were confirmed by the cultures. We took advantages of using Amplicor procedures to perform only one PCR and several probes from the same amplified product.
The Amplicor kit with high sensitivity and specificity seems to be a good complementary diagnostic tool for rapid diagnosis of MAC/MIC infections even in blood samples in VIH patients. However, this study requires a greater number of samples because the prevalence of MAC infections is decreasing due partially to antiprotease therapy.

Evaluation of the LCx MTB assay in pulmonary and extrapulmonary samples

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Introduction: The microbiological diagnosis of *Mycobacterium tuberculosis* (MTb) is based on positive smears and cultures. Both tests have a low sensitivity (S) and smears lack specificity (SP) and culture results are slow.
Aim: Evaluation of LCx™ Probe test for the diagnosis of MTb in pulmonary and extrapulmonary clinical samples comparing with culture results.
Methods: 115 samples with high clinical suspicion of MTb were selected and decontaminated with NALC method a planted on solid selective MTb media, and frozen (- 80°C) until tested with LCx™.
Results: Results (S, SP, Positive Predictive Value (PPV), Negative Predictive Value (NPV)) in relationship with cultures are shown on the table.

TABLE

	S (%)	SP (%)	PPV (%)	NPV(%)
Sputum/Gastric washing (n=45)	90,6	100	100	81
Bronquial aspirate (n= 29)	88,2	100	100	85
Pleural Liquid (n= 16)	38	100	100	27
Biopsies (n= 17)	85,7	100	100	81
Urine (n= 8)	100	100	100	100

In 15 samples (3 positive smears and 12 negative smears) cultures were positive and LCx™ was negative. Inhibitors were discarded in all the samples, amplifying Beneglobine gene.
Conclusion: LCx™ is an authomatic test easy to perform in the Mycobacteria Laboratory. In samples with negative smears the diagnosis of MTb may be advanced for 4 weeks. It is particularly useful namely for the study of respiratory samples, or in those with positive smears and contaminated culture and finally in those with positive smears and high suspicion of atipical mycobacteria.

Comparison of PCR, culture and histopathology for the diagnosis of tuberculous arthritis

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Aim: The utility of PCR for the diagnosis of tuberculous arthritis was compared with conventional methods.
Method: 63 paraffin-embedded synovial biopsies (p.s.b) of patients with tuberculous arthritis (confirmed with positive cultures of synovial liquids or biopsies and/or histopathology) and 29 controls with non tuberculous chronic synovitis were studied with PCR method. Betaglobin gene (BG) was amplified in all samples.
Results: In 36/63 BG was negative and not recovered for PCR study (32 samples ≥ 10 years; 4 samples < 10 years) and therefore 27 samples and 24 controls were selected for the test.

	PCR +	Culture +
Synovial biopsies	14/27	15/17
Controls (n=24)	0	0
Sensitivity	52%	88%
Specificity	100%	100%

Sensitivity of PCR and culture was compared with clinical diagnosis and evolution of patients.
Conclusions: PCR test applied on p.s.b is a rapid, and useful test for the confirmation of tuberculosis in historic samples with negative cultures, or in samples devoid of microbiological specific studies. However compulsory internal control of amplification is needed in all samples.

Evaluation of a molecular biology technique (Ligase chain reaction–LCR) in the tuberculosis diagnosis

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Purpose: Evaluation of the "Ligase chain reaction" (LCR) technique in the tuberculosis diagnosis employing pulmonar and extra-pulmonar samples.
Method: Using as diagnostic approach the positivity in Löwenstein-Jensen (L-J), 73 samples estimated between January and February 1997. From the 73 samples, 63 were of pulmonar origin and the remainder were extra-pulmonar. Baciloscopy was made concerning all the samples, using Ziehl-Neelsen and optic microscope examination. Pulmonar samples were decontaminated and homogeneized by N-acetil-cisteín standart technique. It wasn't necessary to apply decontamination to the non-pulmonary samples.
Two aliquots of each sample were made, one for L-J and another for LCR processing. The remainder steps were subjected to the comercial supplier norms (Abbott®).
Results: The LCR specificity was 100%.
The sensibility of Ziehl-Neelsen, L-J and the LCR technique in respect to the pulmonary samples were respectively 40%, 100% and 95%.
Only one of the extra-pulmonary samples was positive in L-J and LCR, and it had a negative baciloscopy.
Conclusion: The technique assayed had a specificity of 100% and an elevated sensibility, although intermediate Ziehl-Neelsen and L-J.
The LCR main advantage is to provide a rapid tuberculosis diagnosis. Its high specificity and sensability gives it an outstanding place as an alternative diagnosis technique if properly conjugated with the tradicional methods.

Efficiency of the ligase chain reaction test (Abbott LCx) for detecting *Mycobacterium tuberculosis* complex in clinical specimens

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The efficiency of the ligase chain reaction (LCx, Abbott Diagnostics, Abbott Park, Ill.) for detecting *Mycobacterium tuberculosis* complex was evaluated in 209 clinical specimens (pulmonary, n = 151; extrapulmonary, n = 58). Clinical specimens - processed with NALC-NaOH and concentrated by centrifugation - underwent culture in solid and liquid media as well as DNA amplification by the LCx method. Ninety-one specimens were obtained from patients with *M. tuberculosis*-induced disease (including 9 cases with MDR strains); 22 specimens were from patients with MOTT infection: *M. avium* (14), *M. xenopi* (3), *M. kansasii* (2), *M. gordonae* (1), *M. marinum* (1); 96 specimens were negative by culture. The efficiency of a test indicates the percentage of patients correctly classified by the test (diseased and non-diseased). For pulmonary samples, culture sensitivity was 95.7%. The efficiency of LCx was 99.1% with the following calculated values: sensitivity 97.8%, specificity 100%, positive predictive value 98.9%, negative predictive value 99.2%. In the case of extrapulmonary samples, culture sensitivity was lower, i.e. 80%. LCx efficiency was 96.6% with the following calculated values: sensitivity 89.5%, specificity 100%, positive predictive value 94.4%, negative predictive value 97.6%. LCx efficiency was high (96.6%) even in the case of specimens that had been stored at -80°C for 2 to 3 years.
The efficiency of the LCx test was completely satisfactory in the case of pulmonary specimens. The 89.5% sensitivity value obtained in the case of extrapulmonary specimens was not entirely satisfactory; this data however, must be weighed against the 80% sensitivity of the culture method. The good efficiency and rapidity of LCx are particularly appreciable in the case of epidemics due to MDR strains.

Prospective evaluation of Gen-Probe's 2nd generation amplified *Mycobacterium tuberculosis* direct test

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Background: The performance of the 2nd generation of the Amplified Mycobacterium Tuberculosis Direct Test (AMTDT-2; Gen-Probe, San Diego, CA.) for the direct detection of *Mycobacterium tuberculosis* (Mtb) was evaluated by testing prospectively collected respiratory tract samples.
Methods: 1584 respiratory specimens of 704 patients were decontaminated by the sodium dodecyl sulfate-sodium hydroxide method, screened for acid-fast bacilli by fluorescence microscopy, and cultured on Bactec 12B and Lowenstein-Jensen medium. Remnants of the sediments were processed according to the guidelines of the manufacturer. Amplification results were compared to those of culture.
Results: 39 cultures yielded Mtb (prevalence 2.5%). Sixty samples from three consecutive runs had to be excluded from further analysis due to contamination. Compared to culture results, AMTDT-2 and microscopy had a sensitivity of 97.4% and 74.4%, respectively; specificity was 96.2% and 99.1%, respectively.
Comments: AMTDT-2 was very sensitive in detecting Mtb, and proved to be a powerful tool to exclude the presence of Mtb in respiratory specimens: of culture confirmed specimens for Mtb, all smear-positive specimens were detected, and only one (10%) smear-negative specimen was missed. Laboratory reconstruction works resulting in suboptimal environmental conditions probably were a major contributor to the contamination problem. Interpretation of the results should always be done in the context of clinical and sociodemographic data.

Clinical value of rapid molecular PCR testing in TB'S diagnosis

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Tuberculosis (TB) continues to be a worldwide health problem. The early detection of a TB patient is very meaningful, so as to enable starting a specific therapy and avoiding further TB expansion. The traditional culture method take two to eight weeks. Using the amplification methods, such as polymerase chain reactions (Cobas Amplicor), the ligase chain reaction (LCX), or transcription-mediated amplification (TMA, Gene Probe), the detection of TB can be completed in one day. All these methods were evaluated in this study: the Cobas Amplicor, the LCX, and the TMA method showed a sensitivity of 96 %, 94 %, and 86 %, respectively. In comparison, sensitivity of culture methods (Loewenstein and Bactec) was 94 %, that of Ziehl Neelsen 55 %. Specificity for all methods range between 94% and 100 %.

The high clinical value of PCR testing is related to the possibility of early detection and immediate implementation of treatment of TB. In addition, PCR testing is less expensive, and may make additional tests (e.g. computertomography, bronchoscopy) unnecessary. On the basis of our results we are currently performing a large scale prospective study to redefine the diagnostic strategies for TB. Preliminary results recommend molecular PCR testing on patients suspect of TB infection.

Rapid identification of *Mycobacterium genavense*

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Clinical microbiology laboratories may be faced to difficult diagnosis of mycobacterial infection for smear positive specimens which do not yield growth on solid or liquid media. Molecular biology methods may contribute to identify at the species level these difficult to grow organisms. However commercial kits for direct detection in clinical samples are not available for the identification of mycobacteria other than tubercle bacilli.

In the National Reference Center for Mycobacteria in Institut Pasteur we received in 1996-1997 DNA from clinical specimens extracted using the Roche Amplicor kits for TB identification. Extracts were from specimens, all smear positive, which scarcely grew either on charcoal medium or in liquid media (Bactec 12B, MGIT) and were negative by Amplicor test for tubercle bacilli detection. Extracts consisted of specimens submitted to the first steps of the Amplicor protocole, i.e., incubated for 45 minutes at 60°C with lysis reagent and neutralized. Identification was performed by amplification of the *hsp65* gene and restriction enzyme analysis (PRA) of the PCR products, according to Telenti et al (J.Clin.Microbiol, 1993, 31, 175-178). *M. genavense* was identified for all specimens of the 5 patients investigated. These results show that smear positive specimens which turn out to be negative for the presence of tubercle bacilli by amplification methods should be further submitted to additional molecular methods. PRA represents an attractive alternative for this issue.

Increased detection rate of *M. tuberculosis* complex in non pulmonary specimens, using Gentrob amplified *M. tuberculosis* direct test (MTD) as compared to radiometric Bactec culture technique

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Introduction
There is a need for a more rapid and accurate laboratory diagnosis for the detection of *M. tuberculosis* complex in non-pulmonary specimens. The traditional microbiological techniques are relatively insensitive to the small number of Mycobacteria present in these clinical specimens.

Material and Methods
In all 743 non-pulmonary decontaminated specimens were tested with the GenProbe Amplified *M. tuberculosis* complex Direct Test (MTD). This technique utilizes proprietary isothermal transcription-mediated amplification (TMA) of target rRNA, via DNA intermediates, followed by chemiluminescent detection of amplicons with an acridinium ester-labelled DNA probe. The same decontaminated specimens were cultured utilizing the radiometric Bactec culture system.

Results
Of the 743 specimens, 618 were urines, 41 CSF's, 25 pleural fluids, 4 pus, 8 tissues and 47 fluids (other). Culture detected 12 positives and MTD detected 61 positives. The overall sensitivity was 19.6% for culture and 95.3% for MTD. Specificity was 100% for culture and 99.7% for MTD.

Conclusion and Discussion
All positive results were resolved with the clinical diagnosis of the patients. The results demonstrate that MTD is highly sensitive and specific for the detection of *M. tuberculosis* complex in non-pulmonary specimens. The GenProbe MTD, because of its increased sensitivity and specificity, should replace the present microbiological methods which are not capable of detecting small numbers of *M. tuberculosis* complex in non-pulmonary specimens.

Pulmonary infiltrative infection with a new atypical *Mycobacterium* complicating pulmonary sarcoidosis

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A 26 years old patient with pulmonary sarcoidosis and corticoid therapie (40mg/day) was hospitalized for progredient dry coughing accompanied by alternating marked fevers. A chest roentgenogram revealed prominent infiltrations in the right upper lobe. C-reactive protein (> 100mmol/L), blood sedimentation reaction (69/90) with leukocytosis (25 000) were indicative for bacterial infection. Following antimicrobial chemotherapie with initial ofloxazin, later cefuroxime and clarithromycine, no clinical benefit could be noted and the infiltrations worsened with bilateral affection. After repeated culture of a rapid growing mycobacterium with shiny aspect on solid media the patient's fevers promptly decreased as he received an antituberculous combination therapy with clarythromycine (2x500mg), rifampicine (1x600mg)and ethambutole (3x400mg). 4 weeks later the infiltrations also declined significantly. The mycobacterium was cultivated from 7 specimens: 3x sputum, 2x bronchial secretion, 2x bronchial lavage. Resistance testing revealed resistance to isoniazid,ethambutol , rifampicin, streptomycin, pyrazinamide, clarythromycin and trimethoprime/sulfamethoxazol, sensitivity to ciprofloxazin, amikacin and cefoxitin. Histology (lung biopsy): tuberculoid granulomatous tissue reaction with Langerhans cells and intracytoplasmatic acid fast rods without caseation. Biochemical profile: growth at 25°C to 42°

C in 4 days after inoculation. Nitrate reduction +, niacine-, semiquantitative catalase (68ºC) +, Tween hydrolysis 5 days +, arylsulfatase 3 days +, tolerance to 5% NaCl +, susceptibility to T2H (5mg/l)-, acetamide, urease, nicotinamide, pyrazinamide, succinamide, allantoine +, acid production from carbohydrates: mannitol, dextrose, inositol, sorbitol, fructose, rhamnose +, arabinose-, iron uptake +. Alpha-mycolate and methoxy-mycolate positive. Sequencing: new species with similarity to *M. microgenicum*.

Hypersensitivity pneumonitis associated with exposure to *Mycobacterium avium* complex in a home SPA: case reports

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A recent report of a pulmonary illness associated with exposure to *Mycobacterium avium* complex (MAC) in hot tub water raises the question of hypersensitivity pneumonitis (HP) as another expression of pulmonary disease that may be related to this organism.

In April 1997, a 52-year-old livestock farmer was referred with a history of nonproductive cough in the winter of 1995-96, and recurrence in the winter of 1996-97 with associated nodular interstitial pulmonary roentgenographic findings on chest films and subsequent high resolution computed tomographic chest scan typical of HP. His relapses and progressive hypoxemia prompted open lung biopsy which demonstrated acid-fast bacilli (AFB) and pathological changes of HP. Subsequent cultures have documented MAC from lung tissue, patient's sputum, and his home spa water, filter, and personal shower.

Three additional patients have been identified during the past year with MAC recovered at the time of lung biopsy demonstrating HP. Water taken from a personal home spa was positive for MAC in each instance.

These findings support the likelihood that a subacute lung infection may be related to exposure in a spa contaminated with MAC.

Endobronchial lesions in nontuberculous mycobacteriosis: a report of two unusual cases

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Although endobronchial obstruction has been described for *Mycobacterium tuberculosis*, the few previous cases of endobronchial atypical mycobacteria have been reported mostly in HIV-positive patients. We present two unusual clinical cases, including one HIV-negative patient.
Patient 1 was a 33-year-old male, homosexual, treated HIV-positive patient (CD4 = 35/mm3). He was evaluated for persistent cough and fever. Chest CT scan revealed mediastinal adenopathy. Bronchoscopy showed endobronchial lesions in main left bronchus. Biopsies revealed granuloma formation and acid-fast bacilli were found in bronchial washings. Cultures yielded *Mycobacterium avium-intracellulare*. Therapy was initiated with ethambutol, rifabutin, clarithromycin, ciprofloxacin and a third anti-HIV drug. A temporary increase of mycobacterial mediastinal and supra-clavicular nodes occured, contemporaneous of improved immunity (CD4 = 153/mm3), and followed by a progressive regression of bronchial and mediastinal lesions. With follow-up of 18 months, there are no bronchial sequelae.
Patient 2 was a 27-year-old male, HIV negative patient. He was followed-up for a untreated chronic myelopathy. A chest roentgenogram and CT scan revealed a middle lobe opacity. Bronchoscopy showed a granuloma lesion causing obstruction of that segment. Cultures of bronchial suctions yielded *Mycobacterium kansasii*. Treatment with rifampicin, ethambutol, clarithromycin and ciprofloxacin during 16 months was effective. But persistent calcified endobronchial lesion required cryotherapy.
These cases illustrate that nontuberculous mycobacteria can lead to endobronchial obstruction, in HIV-positive or negative patients. Initial bronchial lesions may be mistaken for tuberculosis or bronchial carcinoma. Adequate treatment is effective, but repeat bronchoscopies are necessary to detect residual bronchostenosis.

A case report on transmission of *M. tuberculosis* from a dog to its owner

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Mycobacterium tuberculosis (MTB) is usually considered to be mainly a human pathogen. However, many animals are also prone to tuberculosis and other mycobacterial infections, and may be the source of human infection. We report a case, where *M. tuberculosis* infection was transmitted from a dog to the owner. The transmission was confirmed by typing of the both MTB isolates with the standardized IS*6110* restriction fragment length polymorphism (RFLP) method. It seems likely that the dog contracted the bacterium outside the household, possibly by licking sputum off the street, which resulted in the infection of the submandibular lymph nodes. Because of the fistulated lymph node, myriads of bacteria were likely to have been disseminated to the household. The owner must have contracted the bacilli excreted from the fistulated lesion and developed active pulmonary TB eight months later. It is noteworthy that she was pregnant at the time the TB-diagnosis of the dog was made.
The zoonotic aspect of tuberculosis and the possibility of animal reservoirs of *M. tuberculosis* in the household should be kept in mind by physicians tracing the source of a human infection, and by veterinarians handling their patients, especially if fistulized lymph nodes are present.

Rapid diagnosis of peritoneal and pulmonary tuberculosis using PCR in a patient with chronic renal failure on peritoneal dialysis

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Tuberculosis develops rapidly and dramatically in with immunocompromised patients like those suffering from chronic renal failure that are on peritoneal dialysis. There is a critical need for assays to rapidly detect infection with Mycobacterium tuberculosis and to enable treatement to be started quickly in these patients. A 68-year-old woman with renal failure who had been treated by peritoneal dialysis for the previous 2 years presented with an acute infection, with hyperthermia and severe hypoxemia ; the chest radiograph indicated bilateral miliary.
Bronchoalveolar lavage and peritoneal dialysis liquid were analyzed for Mycobacterium tuberculosis using the AmplicorMTB technique (Roche

Mycobacterium ratisbonense sp. nov., a new cryophilic *Mycobacterium* growing at 10° C

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A novel mycobacterial species was isolated three times from urine of a 17-year-old patient suffering from tuberculosis. Acid-fastness, growth characteristics and biochemical properties clearly demonstrated a novel species.

The rapidly growing, non-photochromogenic strain grows between 10°C to 31°C with an optimum at 25 °C (3 days), and at 37°C (2 weeks).

Susceptibility testing was performed on Löwenstein-Jensen and interpreted by the modified proportion method. The isolate was susceptible to streptomycin, ethambutol, clarithromycin, and ciprofloxacin. It is resistant to isoniazid (1 mg/l), rifampin (32 mg/l), and prothionamide (32 mg/l).

The presence of a novel species was confirmed by determination of the 16S rDNA nucleotide sequence (GenBank accession number AF055331), which is unique. For this new rapidly-growing, cryophilic, nonphotochromogenic mycobacterial species we propose the name *Mycobacterium ratisbonense* sp. nov.

Laboratory diagnosis of *M. xenopi* pulmonary infection – one isolated case in a non – immunocompromised female patient

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Human infections caused by *M. xenopi* have been found since 1960. *M. xenopi* is most frequently responsible for opportunistic pulmonary infections, clinically, radiologically and histologically similar to pulmonary tuberculosis. *M.xenopi* rarely produces non pulmonary lesions in patients who are not immunocompromised. An isolated case of a 30 year old female patient of *M. xenopi* pulmonary infection was detected in our laboratory. The isolation and identification of the etiologic agent was fundamental for TP exclusion. Microscopy, time and growth temperature, macroscopic and microscopic colony morfology and biochemical tests are the basis of the laboratory diagnosis. The aim of this study is to present and select the most important procedings to identify *M.xenopi* from other Mycobacteria of Runyon groups II and III, in order to make it as quickly as possible. Several culture media were used, i.e., Lowenstein-Jensen, Ogawa, Ordinary agar and Middlebook 7H10 agar; three diferent incubation temperatures (30°, 37°, 42°C). Biochemical tests performed were: catalase activity (22°, 68°C), catalase activity > 45mm foam, nitrate reductase and urease activity, tween 80 hidrolysis, iron up take, drug resistance to 1,0µg/ml INH, 10,0µg/ml DNH, 5,0µg/ml EMB, 2,0 µg/ml Tb1, 50,0 µg/ml Tb1 and 5,0µg/ml PAS.

We concluded that bacilli morfology on microscopic observation, growth at 42°C temperature and X-colony morfology in 7H10 are fundamental for first aproach. Susceptibility to 1,0µg/ml INH, 10,0µg/ml DNH distinguishes *M. xenopi* from other potential pathogenic non-photocromogenic species specialy *M. celatum* and *M. avium* complex; 5,0µg/ml EMB plus tween 80 hidrolysis alows diferention from non pathogenic strains of Runyon groups II and III.

Use of glycolipid and phospholipid antigens in leprosy

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The clinical manifestations of leprosy are determined by the cellular immune response of the host. There is a wide spectrum in the host response to *Mycobacterium leprae* which is evident in both clinical, histologic and immunological findings. It was described an inverse relationship between humoral and cell-mediated immunity, which could be ascertained with antibodes against PGL-I, an antigen specific from *M. leprae*, and the Mitsuda reaction.

We intended to verify if this inverse relationship could also be found using less specific antigens extracted from *Mycobacterium tuberculosis*.

To 113 leprosy patients classified according to Ridley and Jopling classification and with a Mitsuda reaction, serology against several antigens: PGL-I, PGL-Tb1, SL-I, DAT, LOS and PIM was performed. The sensitivity calculated for each antigen is shown in Table I:

ANTIGEN	SENSITIVITY (%)
PGL-I	52.4
PGL-Tb1	[illegible]
SL-I	4.8
DAT	37.8
LOS	24.2
PIM	56.9

We could also verify that the inverse relationship between humoral and cell-mediated immunity, established with antibodies against PGL-I, was also found for antibodies against PIM and LOS.

Serological detection of TB

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The objectives of the research were to develop simple, easy to use and rapid Enzyme Immunoassays (EIA) for the detection of Tuberculosis. Four seporate EIA's have been developed by Omega Diagnostics. The tests use the highly specific recombinant 38 and 16 KDa antigens and a highly purified lipopolysaccharide similar to LAM. Tests have been developed which are specific to the *Mycobacterium tuberculosis* complex and screening assays which detect all *Mycobacterium* species.

The EIAs all have coloured, working strength reagents for ease of use and take only 105 minutes to obtain a result. A choice of qualitative or quantitative protocols are available to give the user maximum choice.

Ongoing evaluations indicate traditional methods of diagnosis combined with serological testing significantly increase sensitivity. Data from Europe have so far found an increased sensitivity to 85%. Results from these evaluations will be presented.

HLA-DR antigens in pulmonary tuberculosis in Polish population

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The aim of this study was to analyse association between HLA class II antigens and tuberculosis in Poland. HLA -DR antigens were studied in 26 patients with newly detected pulmonary tuberculosis and 58 healthy volunteers.

Histocompatibility typing was performed by PCR-SSP method with primers from Dynal company. For statistical analysis $\chi 2$ test was used after Yates' correction. The probability values were corrected for the number of antigens tested (pc) by multiplying them by the number of antigens tested. The relative risk (RR) was calculated by Woolf's method.

We found that: HLA- DR16(2) $p<0,001$; $pc<0,02$) appearance was significantly higher in patients with tuberculosis than in the tested group of healthy controls. The highest relative risk of tuberculosis was connected with DR16(2) (RR=9,7). The appearance of antigen HLA-DR13(6) ($p<0,001$; $pc<0,002$) was significantly lower in pulmonary tuberculosis (with relative risk RR<1) than the tested group of healthy people.

The results obtained can suggested that: 1.Presence of HLA- DR 16(2) can extend the risk of development tuberculosis. 2.HLA- DR13(6) appearance was significantly more rare in pulmonary tuberculosis than in group of healthy people, the relative risk (RR) <1 can be connected to their relation with the genes of susceptibility to tuberculosis.

Kinetics of production of TNF-α and TNF-α receptors in *Mycobacterium avium*-infected BALB/C mice

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BALB/c mice intraperitoneally infected with 10^5 *Mycobacterium avium* CFU (strain 485, transparent colonies) were studied for *M.avium* growth in spleen and for production of TNF-α and TNF-α receptor (RI and RII in vivo. TNF-α and TNF-α Rs mRNAs were monitored in spleen by reverse-transcription PCR and TNF-α protein and soluble (s) TNF-α Rs were assessed in blood by ELISA/cytolytic assay throughout a 70-day experimentation period. In spleen, after a first phase of infection associated with a 2 log increase in the CFU number up to day 21, a second phase occurred, with the bacterial burden remaining fairly constant in the subsequent observation period. Around 10^2 CFU/ml were found in blood between day 1 and 14, followed by a low level bacteremia thereafter. In spleen, TNF-α and TNF-α RII mRNAs increased 4 and 7 times, respectively, on day 21, and then slowly decreased with time; in contrast, TNF-α RI mRNA did not significantly increase at any time in infected organs. The level of sTNF-α in blood increased from day 14 through day 24 and then sharply decreased; more than 90% circulating TNF-α was biologically inactive. While sTNF-α RII peaked around the third week, no increase in sTNF-α RI was observed at any time. These data seem to indicate that the containment of bacterial growth on day 21 correlates with local activation of the TNF-α/TNF-α RII system in spleen. Both molecules appeared to be regulated not only at the transcriptional level, but also post-transcriptionally, as suggested by the concomitant increase of sTNF-α RII in blood; in contrast, no transcription of TNF-α RI mRNA, nor shedding of its soluble forms in blood were observed.
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38 kD ISCOMS: Immune responses to the 38 kD lipoglycoprotein from *Mycobacterium tuberculosis*

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Introduction. Induction of cellular immunity, covering both CD4+ and CD8+ T-cell activation, is a prerequisite for providing protection against infection with *Mycobacterium tuberculosis*. Immunestimulating complexes (ISCOM) are capable of inducing both cellular as well as humoral immunity against protein antigens. In order to incorporate proteins into ISCOMs often lipid tails have to be coupled to the protein. In this study, the immunity against the whole recombinant 38 kD lipoglycoprotein from *M. Tuberculosis* was investigated. The use of different amounts of N-palmitoyloxysuccinimide (PA) and Quil-A and its influence on the immunogenicity of the 38 kD ISCOMs was also studied.
Methods. Lipid tails were attached to the 38 kD lipoglycoprotein of *M. Tuberculosis* using different concentrations of PA. After purification, ISCOMs were prepared by the dialysis method using different concentrations of Quil-A. The products were analyzed for protein and free Quil-A content. Female C57BL/6J (H-2[b]) mice were immunized s.c. with the 38 kD ISCOM at day 0, 10 and 20. The anti- 38 kD antibody response (IgG) was studied in serum by ELISA. The capacity of the ISCOMs to induce cytotoxic T- cells (CTL) was also analyzed in a standard ^{51}Cr release assay, performed after *in vitro* restimulation of the primed spleen cells.
Results. The use of higher amounts of Quil-A during the ISCOM preparation increased the amount of protein incorporated into the ISCOM, whereas the use of higher amounts of PA did not influence this parameter. Low antibody response were observed after primary immunization with the 38 kD ISCOM which strongly increased after booster injections. CTLs were only induced after the booster injections. The higher concentrations of Quil-A seems do not affect the immunogenicity of the 38 kD ISCOMs. However, the increase of PA used for the preparation of ISCOMs decreased its immunogenicity.
Conclusions. The optimal method for preparing ISCOMs containing the 38 kD mycobacterial lipoglycoprotein consists of the use of low amounts of PA. The increase of lipid tails attached to the 38kD lipoglycoprotein probably modifies some important epitopes.

Downregulation of Interferon-gamma and Interleukine-2 in HUMAN PBMC upon exposure to glycopeptidolipids and total Lipids from *Mycobacterium avium*

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TH1-type responses are important in host immunity against the infections caused by M. avium as well as HIV. Previous studies have suggested that substantial quantities of bacterial lipids may accumulate and persist within host cells during chronic stages of M. avium infections. Recent studies with human T-cell lines have demonstrated that M. avium lipids can affect TH1-type responses such as IL-2 and IFN-g. In this study, human PBMC from healthy donors were exposed to total lipids and serovar-specific glycopeptidolipids (GPL) extracted from M. avium serovar 4 and 8. Exposure to the total lipids from both serovars significantly suppressed PHA/PMA-induced secretion of IL-2 and IFN-g as determined by ELISA. Among GPL antigens, serovar-4 specific lipids were more efficient in inhibiting the TH1-response than the serovar-8 specific lipids. The above observations were also repeated on CD4+ T-lympocytes obtained by PBMC enrichment, and further confirmed that M. avium lipids suppressed the TH-1 response. The suppression of TH-1 response was also investigated at the transcriptional level by performing reverse-transcriptase-PCR which confirmed that a significant proportion of the TH1-type response inhibition was observed at the transcriptional level. The results obtained provide an interesting insight in the pathogenesis of opportunistic M. avium infections in terminal AIDS patients.

Postantibiotic effects of clarithromycin, clofazimine, rifampin, sparfloxacin and amikacin against *Mycobacterium avium* in human macrophages

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Amikacin, sparfloxacin, ethambutol and clarithromycin are potential anti-Mycobacterium avium drugs. As the clinical efficacy of each drug depends both on pharmacocokinetic/dynamic parameters dictating the time course of its concentration at the site of infection and its antimicrobial effect per se, we investigated on the "Postantibiotic Leukocyte Enhancement (PALE)" and "Pulsed Exposure (PE)" effects of the above drugs against M. avium in a human macrophage model. This study was meant to determine the possible cummulative intracellular postantibiotic effects (PAE) due to resulting synergistic interactions between the drug, the macrophage, and the bacterium. In the first case (PALE) M.avium was treated by antibiotics prior to its phagocytosis, whereas in the second case (PE), antibiotics were added after M.avium phagocytosis. In both cases, the drugs were used at their peak serum level concentrations (Cmax), and the time of drug-contact was limited to 2h. The results obtained underlined two different patterns ; for pattern I drugs PE was significantly higher than PALE, whereas on pattern-II profile, PALE was superieur to PE. The prolonged and persistent effect on intracellular M. avium inhibition of a single 2h exposure to some of the drugs suggests the possibility of an intermittent administration of selected drugs in M. avium infected AIDS patients, who are over-burdened with too many drugs given for various opportunistic infections.

Human T cells recognize MPT64 and its peptides in association with multiple HLA–DR molecules

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The secreted Mycobacterium tuberculosis protein MPT64 is an antigen with potentials in tuberculosis diagnosis because its gene is lacking in several Mycobacterium bovis BCG vaccine strains. To identify the immunogenic regions of the MPT64 antigen, overlapping synthetic peptides were used in this study to map the T cell epitopes by testing peripheral blood mononuclear cells and T cell lines from tuberculosis patients and healthy subjects for proliferation and IFN-γ secretion. The results showed that T cell epitopes were scattered throughout the MPT64 sequence. MHC restriction analysis allowed the identification of N and C-terminally located promiscuous peptide epitopes presented to T cells in association with a large spectrum of HLA-DR molecules. In addition, MPT64 reactive T cells were cytotoxic for monocytes pulsed with the complete protein as well as specific peptides. In conclusion, our study demonstrates that multiple T cell epitopes are distributed along the entire sequence of MPT64 antigen of which some are presented by multiple HLA-DR molecules. These results suggest that the MPT64 protein and some of its epitopes are relevant to medical applications in HLA heterogeneous populations.

Recombinant 38 kDa protein and a higly purified antigen from *Mycobacterium* t. in Elisa serodiagnosis of tuberculosis

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In extrapulmonary tuberculosis or in small children, the approach to the diagnosis with immunoserology may be considered helpful.TB serodiagnosis has been performed since years with IgG, IgM and IgA tests utilizing different mycobacterial antigens. Two mycobacterial tests produced by OMEGA Laboratories (Alloa,Scotland,U.K.) were performed in our laboratory.The first test,Pathozyme-Myco IgG,IgM, IgA,utilizes a mixture of two antigens: one extracted and purified from Mycobacterium T,the other a recombinant 38 kDa antigen from E.coli,higly specific for M.Tuberculosis complex.
The second test, Pathozyme TB complex IgG, utilizes only the recombinant 38 kDa protein.
In our study we tested 80 subjects,40 patients with active pulmonary TB,20 with extrapulmonary tuberculosis,10 TB contacts and 10 blood donors.The positivity percentages are the following:

	ACTIVE TB	EXTRAPULM.TB	TB CONTACTS	BLOOD DONORS
N° cases	40	20	10	10
PATH.MYCO-IgG	85%	85%	20%	10%
PATH.MYCO-IgM	35%	45%	20%	20%
PATH. MYCO IgA	87%	45%	10%	10%
PATH TB compl	40%	20%	0%	0%

SENSITIVITY:Path Myco IgG and IgA results show a good sensitivity, both in active and extra pulmonary TB.Path TB complex IgG results give a lower percentage of positivity
SPECIFICITY:IgG, IgM,IgA antibodies to Path Myco antigens are detected in 10-20% of TB contacts and blood donors.Path TB -complex has a higher specificity.
In conclusion, the OMEGA tests performed show a higher sensitivity with a mixture of two antigens. The recombinant test appears higly specific, but lacks of sensitivity.

Effects of high intensity/low frequency noise in the reactivity of pleural milky spots (Kampmeier's foci) by mycobacterial infection

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Milky Spots (MS), also known as Kampmeier's foci, were first described by von Recklinguasen in 1863 and are confined to the parietal pleura leaflet (retrocardiac pleural folds). Previous studies have made clear that pleural MS are immunological fully competent structures that can protect the pleural space from noxious agents, increasing either in number or in size, as a reaction to infection. It is also known that low frequency noise can cause morphological and physiological changes in a number of organs and systems of living organisms, for instance, induces morphofunctional changes on the pleural mesothelium and depression of the immune system.
In our experimental protocol, 20 Wistar rats were used to study the structural changes of pleural milky spots, induced by mycobacterial intrapleural infection while in a stressfull environment. The animals were sacrificed 21 days after infection and sections of retrocardiac pleural folds were observed using a light microscope. The sections were stained with the Ziehl-Neelsen method, to visualize the acid fast bacilli.
The structural features that we have observed by light microscopy shown a hipercellularity of the pleural MS of rats infected with *M. avium*, , due to the increase of the number of mononuclear cells. However this increase was different according to the length of time of exposure to high intensity/low frequency noise. The animals submitted to more hours of noise stress had a smaller increase in the width of the pleural MS and a less evidence of cellular infiltrate of mononuclear cells.
In conclusion, the response of pleural MS to mycobacterial infection is compromised if the infected host is chronically exposed to high intensity/low frequency noise and therefore immunologically depressed. These changes in the pleural MS reactivity seem to have correlation with the duration of exposure to noise stress. These findings are in accordance with the high occurrence of cardiopulmonary diseases in patients who belong to high risk groups in stressfull environment, like workers of aeronautical industries.

Reduced T-cell responses to virulent *M. tuberculosis* in pulmonary tuberculosis patients

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Objectives: To assess the role of T-cell proliferation, activation and cytotoxicity towards purified protein derivative (PPD), *Mycobacterium tuberculosis* H37Rv (Mtb) and heat-killed *Mycobacterium tuberculosis* H37Rv (Hk Mtb) in healthy donors and pulmonary tuberculosis patients.

Methods: Blood was collected from 1) healthy BCG vaccinated individuals and 2) pulmonary tuberculosis patients. Standard lymphocyte proliferation, ^{51}Cr cytotoxicity assays, flow cytometry and ELISA techniques were used. Briefly, peripheral blood mononuclear cells ($1x10^6$/ml) were stimulated with PPD, *Mycobacterium tuberculosis* H37Rv and heat-killed *Mycobacterium tuberculosis* H37Rv for 6 days and used in assays.

Results: BCG vaccinated individuals demonstrated extensive proliferation and IFN-γ production when compared to the pulmonary tuberculosis patients. Stimulated lymphocytes from tuberculous patients showed significantly reduced response to virulent Mtb and the Hk Mtb preparation. Pulmonary tuberculosis patients showed no difference in IL-5 production when compared to the healthy individuals. Comparisons of the cytotoxic activity between healthy donors and tuberculous patients showed that patients demonstrated a reduced ability to lyse antigen pulsed target macrophages. T cell activation profiles were also found to be reduced towards all the antigens when compared to the control group.

Conclusions: Pulmonary tuberculosis patients therefore have reduced capacities to activate and lyse virulent *Mycobacterium tuberculosis* infected target macrophages.

Interaction beteween mycobacterial glycolipids and human neutrophils

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Mycobacterium tuberculosis and several other mycobacteria are intracellular pathogens i e they can survive and multiply within phagocytes This capacity might e g be due to mycobacterial surface molecules such as the complex cell wall glycolipids A microtiter based technique was developed in order to study the interaction between mycobacterial cell wall lipids and phagocytes Human neutrophils were allowed to interact with purified mycobacterial glycolipids and the production of reactive oxygen radicals was followed with a chemiluminescence technique Phenolic glycolipids (PGLs) from four mycobacterial species were analysed and it was shown that PGLs from *M.tuberculosis* and *M.kansasii* induced an oxidative burst in neutrophils, while PGLs from *M.marinum* and *M.bovis* BCG did not Intact sugar-structures were shown to be essential for the activation. The PGLs from *M.tuberculosis* and *M kansasii*, which are capable of activating the neutrophils, have three and four sugar units respectively, while the PGLs from *M marinum* and *M bovis* BCG, not able to activate the neutrophils, have only one sugar unit each The results indicate that several sugar units are required for sufficient binding and activation of the neutrophils.

Extended major histocompatibility complex haplotypes in pulmonary tuberculosis

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The polymorphism that characterizes HLA molecules resides upon variability within the chains of the receptor for processed antigen peptides as well as for recognition of the T-cell receptor, and determines peptide specificity and interaction with T cells. As far as tuberculosis is concerned, it is reasonable to assume that differences observed in the HLA allele and haplotype frequencies may relate to progression to disease within an infected population and also that this relationship may be based upon differences in the nature of processed mycobacterial peptides.
In a previous study, the authors have observed a significant decrease in the HLA-DRB1*15, -DQA1*0102 and -DQB1*0602 association in controls with low-interpretative-associated tuberculin reactivity, as compared to tuberculous patients. The present study aimed the evaluation of the contribute of MHC haplotypes, namely those associated with HLA-DRB1*15, probably associated to susceptibility to disease progression after *Mycobacterium tuberculosis* infection, highlighting the importance of the individual genetic characteristics in infection and disease.
Ninety-five non-multitransfused pulmonary tuberculosis patients and 36 infected, non-BCG immunized, tuberculin positive controls were typed for HLA-A, -B, -Cw, -DRB1, -DQA1 and -DQB1 by PCR-based DNA amplification methods. In addition, several MHC complement components (BF, C2, C4A and C4B) polymorphisms was studied by high-voltage electrophoresis and isoelectric focusing.
Estimation of maximum-likelihood multi-locus haplotype frequencies from genotypic data was computed using an expectation-maximization algorithm.
Two major extended haplotypes were found in pulmonary tuberculosis patients, HLA-A*03-B*51-Cw*0702-BF*S-C2*C-C4A*3-C4B*1-DRB1*15-DQA1*0102-DQB1*0602 and HLA-A*02-B*51-Cw*1502-BF*S-C2*C-C4A*3-C4B*1-DRB1*15-DQA1*0102-DQB1*0602, accounting for the previously observed association with HLA-DR15. Both haplotypes were found common among European Caucasoid populations. The analysis of the control group revealed two extended haplotypes, HLA-A*02-B*51-Cw*1602-BF*S-C2*C-C4A*3-C4B*1-DRB1*15-DQA1*0102-DQB1*0602 and HLA-A*2501-B*18-Cw*1203-BF*S-C2*C-C4A*3-C4B*1-DRB1*15-DQA1*0102-DQB1*0602, but, according to the previously noticed decrease in the HLA-DR15-DQA1*0102-DQB1*0602, a larger number of HLA-DR15 infected controls should be studied for reasonable comparisons.
These results suggest the hypothesis that an immunogenetic constellation involving an extended HLA-DRB1*15 haplotype contributes to the different courses of tuberculous infection.

Epidemiological study of paratuberculosis in ruminants in Alentejo Portugal

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Specific objectives.
- Cross-sectional study of Ruminant Paratuberculosis in the region of Alentejo.
- Construction of 3 vulnerability (low, middle and high risk) profiles in accordance with the presence/persistence of the infection and management practices.
- Testing and validation of a PCR method for the IS specific diagnosis and comparative study with others diagnostic tests: bacterioscopy, bacteriology, and serology.
Material and methods.
- Target population: 7.233 bovine cattle herds and 13.040 small ruminants in Alentejo
- Epidemiological unit: Dairy or beef herd, flock (sheep or goat).
- Sample size: 365 cattle herds and 374 flocks of small ruminants =17.793 samples.
- For serological screening, a stratified random sample from the brucellosis eradication program were analyzed in CFT and ELISA. Proportional allocation was used to decide upon the number of sampling units in each stratum.
- Bacteriological and PCR tests will be carried out on samples collected from clinically and serologically positive animals.
- A questionnaire was submitted by field Vets recording information about the production system, epidemiological scenarios and disease profiles.
Preliminary Results
- Estimates of prevalence (clinical and subclinical paratuberculosis) will be presented for an initial group of 108 herds/flocks investigated.
- Kappa statistic will be used to measure agreement between CFT and ELISA.

Detection by Polymerase Chain Reaction of *Mycobacterium avium* subspecies *avium* in formalin-fixed, paraffin-embedded tissues of captive exotic birds (1983-1997)

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A presumptive diagnosis of avian tuberculosis can be made when an avian tissue has characteristic histopathologic lesions along with acid-fast bacilli. However, a definitive diagnosis requires isolation of the causative organism, a process that can take several weeks to complete. The purpose of the study was to determine whether formalin-fixed, paraffin-embedded, archival avian tissues could be tested by polymerase chain reaction (PCR) to provide a reliable and more rapid diagnosis of avian tuberculosis. Tissues were examined from both presumptive and definitive cases of avian tuberculosis from captive exotic birds covering a 14 year period. The primers used for PCR amplified a 160-bp fragment of 16S rRNA, a sequence specific for both *Mycobacterium avium* subspecies *avium* and *Mycobacterium avium* subspecies *paratuberculosis*. If a detection was made on a sample, it was presumed that *M. avium* subsp. *avium* was the organism being detected. The PCR detected the sequence in 26 of the 97 samples (27%). Some of the negative PCR results may be explained by any of several factors that adversely affect nucleic acid integrity, particularly prolonged fixation in formalin. Of the samples that were culture positive for M. avium and were known to have been fixed in formalin for four weeks or less, PCR detected 11 of 17 samples (65%). The findings of this study demonstrate that PCR can be a rapid indicator of the presence of *M. avium* subsp. *avium* in formalin-fixed, paraffin embedded tissues; however, the low detection rate in this sample set may limit its practical use as a diagnostic tool.

Isolation of *Mycobacterium africanum* from a bull in Northern Bavaria

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Mycobacterium africanum is a pathogen found in tuberculosis patients in certain parts of Africa and is a member of the *Mycobacterium tuberculosis* complex. Sporadic occurence of *M. africanum* infections in various regions of Europe have been reported recently. In the German district of Bavaria, one to three infections of humans caused by *M. africanum* are observed annually. However, only very few is known about the occurence of *M. africanum* in animals.
Here we describe for the first time the isolation of *M. africanum* from a mediastinal lymph node with tuberculosis alterations of a young bull from a herd of cattle in Northern Bavaria. Epidemiological investigations using IS6110 RFLP-typing demonstrated that the source of infection was probably a member of the family of the owner of the animals.

Use of restriction fragment length polymorphism for typing *Mycobacterium avium* strains isolated from animals in Slovenia

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Restriction fragment length polymorphism techniques (RFLP) was used to analyse *Mycobacterium avium* complex strains isolated from various animals in Slovenia. All the strains were serotyped. Polymerase chain reaction (PCR) analyses have shown the possession of insertion sequence IS901 in all tested strains. Genomic DNAs were isolated and digested with endonuclease *Pvu II*. The obtained DNA fragments were separated on agarose gel, transfered to a membrane and hybridised. As a probe we have used 1108 bp PCR product specific for the IS901. RFLP analysis was performed using ECL detection system. We have tested 50 samples of DNA obtained from pig, poultry, crane, parrot, cattle and human. The obtained results indicate that in Slovenia we have at lenst six different RFLP types of *Mycobacterium avium*. Our results indicate that RFLP analysis is useful tool for the epidemiologic studies *Mycobacterium avium* infections in animals.

Occurrence of bovine tuberculosis in animals and humans in the Czech Republic in years 1969 to 1996

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As of 10 October, 1968, bovine tuberculosis in cattle was eliminated in the Czech Republic within the framework of national elimination programme (1959 to 1968). The postelimination period (1969 to 1996) was typical by vanishing of infection source reservoirs during several following years. Currently only sporadic case are recorded. In the period of years 1969 to 1996 bovine tuberculosis was newly detected in 369 farms of cattle (42 small farms with up to 9 dairy cows and 327 larger farms with more than 10 dairy cows). No occurence of bovine tuberculosis was found in years 1981, 1987 to 1990, 1993 and 1996. In the remaining years of the period between 1980 and 1996, there were always maximum 3 outbreaks of bovine tuberculosis in cattle detected per year. The rate of infected animals out of the total size of herds was very small and did not exceed 5 to 10 % of animals. In years 1970 to 1996 the infection with *Mycobacterium bovis* was also diagnosed in totally 119 animals (zoological gardens, wild animals, small farms) and in 10 samples of milk. In the sense of OIE definition (International Animal Health Code) the territory of the Czech Republic is free from bovine tuberculosis (prevalence up to 0.2 % of infected herds of cattle). In human population in years 1969 to 1996 the spread of *M.tuberculosis* was recorded in totally 77.739 newly infected persons and the infection with *M.bovis* in 476 patients. In 1981 to 1996 the prevalence of bovine tuberculosis ranged in absolute figures between 4 and 20 patients of higher age groups above 50 years (0.04 to 0.20 per 100.000 inhabitants). The incidence of this disease in absolute figures was 3 to 19 patients (0.05 to 0.18 per 100,000 inhabitants).

Some unusual localization of animal tuberculosis

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We propose to present some unusual cases of animal tuberculosis.

This situation was detected in the routinely sanitary inspection, in healthy animals, that were slaughtered in the regional abattoire. This sanitary unit don't have nor microbiological nor anatomopathological laboratories, so in case of suspicious or doubt, the veterinary inspectors send samples of suspected material to the University laboratories. In the cases that we present here, the material was, unfortunatly, sent in formaldeid, leading microbiological tests impossible.

The first situation was observed in an apparently healthy swine. This animal presented only a multinodular spleen, no other lesions were detect.

The second situation was observed in three bovines from different farms. These animals had a single lesion in the popliteum lymph node. As in the first case, no other lesions were detect.

The histological image of all these cases, were a granuloma with lymphocytes, macrophages, epithelioid cells and multinucleated giant cells, Langhans type. In some granulomas, caseation necrosis was observed and some of them with dystrophic calcification. Fast acid bacillus was observed inside the macrophages.

Tuberculosis compatible lesions (*post mortem*) in cattle

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Bovine Tuberculosis is one of the most important contagious diseases, caused by *Mycobacterium* spp., mainly *Mycobacterium bovis* and less often *Mycobacterium tuberculosis* and *Mycobacterium avium*. Due to its economic and Public Health importance, this disease continues to bring out a great interest.

In terms of bovine Tuberculosis eradication program, 82 animals reactive to the tuberculin test were slaughtered. All animals were *ante and post mortem* examinated. The aim of this work, was to determinate which of these animals showed *post mortem* lesions compatible with bovine Tuberculosis and the kind of lesional feature observed.

55 (67%) animals showed *post mortem* lesions compatible with bovine Tuberculosis, occurring as digestive and intestinal primary complex or generalised process. Both were often accompanied by cachexy. These animals were considered inappropriate for human consumption.

Samples of the affected organs from the animals with compatible lesions were collected, fixed in 10% buffered formalin and subject to histopathological examination. All samples were embedded in paraffin, sectioned at 3 µm and stained with heamatoxilin and eosin. It was also subject to a Ziehl-Nielsen specific coloration.

Microscopically, it was observed granulomas with caseation necrosis and dystrophic calcification surrounded by macrophages, epithelioid and Langhans giants cells. The Ziehl-Nielsen coloration showed acid-fast organisms (bacillus).

Detection of IS901 and flanking region in the strains of *Mycobacterium avium complex* isolated from swine in the Czech Republic from 1996 to 1997

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In our previous research in the strains of *Mycobacterium avium* complex, isolated from animals and birds, we detected a correlation between the presence of IS901 and virulence for poultry in more than 90%. Accordingly, 614 strains of *Mycobacterium avium* complex were isolated from swine in 245 herds from 68 districts and examined by PCR method for the detection of IS901 and flanking region 300pb (FR300). We found out that 32.2% of the strains contained IS901, 30.9% had FR300 and 16.9% contained neither IS901 nor FR300. We have also analysed the place of origin of these strains. Out of 74 districts in the Czech Republic, we examined strains isolated from swine herds in 68 districts. Throughout 1996 and 1997, 245 swine herds were positive for *M. avium* complex. The results were as follows: 28.6% of the herds were infected with strains containing IS901, 40.0% were infected with strains containing FR300, 9.8% were infected with strains free of IS901 and FR300, and 21.6% were mixed infected with strains with IS901 and other types. Based on the strains history, we could also compare the prevalence of each type of strain in 1996 and 1997. In both years it was confirmed, that the proportion of isolated strains and that of individual herds infected with the above mentioned strains of *M. avium* complex was almost the same. In 1996 and 1997, the percentage of herds infected with strains containing IS901 was 50.8% and 48.6%, respectively, and that of herds infected with strains containing FR300 was 17.9% and 45.6%, respectively. The occurrence of strains of *M. avium* complex in swine herds in the Czech Republic remains under investigation.

PCR and Elisa test applied to paratuberculosis diagnosis in early stadies of infection in cows

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PCR tecnique and Dot Blot hybridization test were performed on milk, faecal and blood samples, in order to make paratuberculosis diagnosis in animals during preclinical study of infection. 110 animals were examinated in three farms located in Sicily. Biomolecular tests were performed and were compared with an indirect ELISA test IDEXX routinely used in serological diagnosis. For PCR test specific oligonucleotide primers were chosen inner to IS 900 sequence to amplify a 400-bp fragment. A 229-bp was designated by nested PCR from the first amplification product and after being labeled was employed in Dot Blot hybridization test. Sensitivity and sensibility of the methods were established. Negative samples in the ELISA test resulted positive in PCR and some cases of cross reaction were also detected. Biomolecular tecnique may be utilized as tool for validation of paratuberculosis diagnosis with different methods and for detection of latent forms of desease, avoiding the spread of infection in the farms.

Identification and spoligotyping of *Mycobacterium bovis* strains isolated in Italy from different animal species and man

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We have identified eightyfive *M. bovis* strains isolated from bovine, swine and humans by traditional microbiological methods and by two PCR assays developed for species differentiation, one based on a OxyR gene PCR/RFLP to distinguish bovis from tuberculosis (1) and one performed by amplification of the RD1 genome region (2) deleted in M. bovis BCG (3).
In addition we have used spoligotyping (4) to characterize *M. bovis* strains; patterns were obtained by hybridization of digoxigenin labelled amplified DNA with multiple spacer oligonucleotides in microtiter plates (PCR-ELISA).
Preliminary results revealed that *M. bovis* strains isolated in Italy are distinguished into 18 spoligotypes one of which represented by four strains which were collected from the same livestock at different time. Two types were described previously by Gutierez et al. (5) respectively in seven and two isolates of human origin. Further results will be discussed.

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Zoonotic transmission of *M. microti* and *M. avium*

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As a result of the comparison of DNA fingerprinting results of *Mycobacterium microti* isolates from animals and *M. tuberculosis* complex isolates from humans, the first four human cases caused by this mouse-pathogen were diagnosed. All *M. microti* isolates were found to contain a short direct repeat region, giving an exceptional two-spacer pattern in 'spoligotyping'. DNA fingerprinting enables a reliable recognition of this uncommon species, usually not suspected to cause disease in humans.
Among the non-*M. tuberculosis* complex strains, *M. avium* complex (MAC) is the most frequently isolated species in the Netherlands, 218 out of 420 (52%) in 1996. In recent years, IS1245-based restriction fragment length polymorphism (RFLP) typing has become available for *M. avium* isolates. RFLP typing proved to be more discriminatory than serotyping. Although the clinical relevance of *M. avium* infections is not always clear, our results have shown that successive *M. avium* isolates of individual patients, during periods up to two years, had identical fingerprints. This indicates that these patients were colonized- or infected with particular *M. avium* strains for longer periods. The source of human *M. avium* infections is still unclear. All strains isolated from 23 different bird species without exception showed a particular 3-band IS1245 RFLP pattern. All of these 'bird type' isolates also showed a specific, highly conserved, IS901-associated RFLP. This indicates that the bird isolates represent a separate taxon within the *M. avium* complex. The 'bird type' pattern was not observed among any of 191 human MAC isolates tested, ruling birds out as significant sources of infection for humans. A high degree of similarity was observed among MAC isolates from pigs and humans. Of 81 pig isolates typed, 59% were classified in common IS1245 genotype families with human isolates.

Nontuberculous mycobacteria in cystic fibrosis patients

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In patients with cystic fibrosis (CF), the major cause of death is the progressive pulmonary disease with infections due to *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*. Pulmonary colonization and infections by mycobacteria have recently been recognised as a potentially important clinical problem. In a nine-month-period, we investigated about 40 patients with cystic fibrosis from a CF clinic center and we screened their respiratory secretions for mycobacteria. The specimens were decontamined using N-acetyl-cystein-NaOH method followed by 5% oxalic acid to reduce the incidence of Pseudomonas overgrowth. Five patients had positive culture, one positive smear. The mycobacteria isolated were *M. avium* complex (once), *M. chelonae* (twice), *M. abcessus* (twice). These patients were clinically considered to be infected and they received antimycobacterial drugs.

Setup of a procedure for isolation of mycophages from environmental and clinical samples

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As the other microorganisms of special interest for public health, the use of phages for typing of *Mycobacterium tuberculosis* was considered a fruitful technique to establish epidemiological connections among cases which were apparently unrelated, and to confirm the common origin of an outbreak. Interesting investigations were carried out on strains of *M. kansasii* which were isolated both from urban water-supplies and from infected users. The possibility of using phages also proved to be a promising criterion for *M. avium* typing, in addition to serogrouping. We firmly believe that the use of mycophages can be a further subject of investigation in the mycobacteriological field, both as epidemiological and ecological markers. For this reason, we are setting up a procedure for their isolation using *M. smegmatis* ATCC 607 as propagating strain.
Tissue culture flasks (surface 120 cm²) containing a layer of glycerolated trypticase soy agar and 50 ml of buffered nutrient broth were spiked with a mix containing the sample (alternatively represented by 5 g of faeces, 5 ml of blood, 50 ml of surface water, or 5 ml of garden soil) and with 1 ml of broth-culture of ATCC 607. Incubation was performed at 37 °C keeping the flasks slant-wise in order to get different gradients of aeration. After a two-day incubation and an overnight halt refrigerator, the liquid medium was centrifuged and filtered using a 0.22 µm membrane (Millipore). The lytic activity was evaluated by spotting method on agar plates inoculated with the strain ATCC 607. When lysis appeared, a confirmation by dilution was performed. At the moment we have isolated fourteen mycophages only from environmental samples; they appear different on the basis of their host range.
Once our preliminary study has been performed, we would like to attempt a phage typing approach on *M. avium* strains related to AIDS cases.

Mycobacteria in water and biofilms in drinking water systems

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In order to determine whether drinking water and drinking water systems in the United States contained mycobacteria, *Mycobacterium* spp. were enumerated and identified in 8 water systems over an 18 month period. The systems differed in raw water source (i.e., surface or well), organic matter content (i.e., high, moderate, or low), and method of disinfection (i.e., chlorine, chloramine, and ozone). Samples of raw and post-disinfection waters were collected as well as samples collected at the midpoints and ends of the distribution systems. This is a preliminary report of the results to date. Compared to other methods, 0.05% cetylpyridinium chloride disinfection and plating on M7H10 agar medium containing 0.5% glycerol and 10% albumin-oleic acid resulted in the highest numbers of *Mycobacterium* spp. Biofilm samples were collected from the surfaces inside water meters or pipe sections by scraping a 4 cm^2 section and suspending the material in water. *Mycobacterium* spp. isolates (including *Mycobacterium avium*) were recovered from 56% of raw, 31% of post-disinfection, 25% of midpoint, and 56% of dead-end water samples. Average *Mycobacterium* spp. CFU/ml sample were: 100 for raw, 35 for post-disinfection, 70 for midpoint, and 170 for dead-end water samples. Thirty percent (90%) of biofilm samples yielded *Mycobacterium* spp. (range 11 - 544 CFU/cm^2). Cells of *Mycobacterium avium* survived and grew within the phagocytic protozoa *Tetrahymena pyriformis* and *Acanthamoeba polyphaga*. Based on that observation, these phagocytic protozoa were added to water samples to determine if they could enhance recovery of mycobacteria. Following addition and a 10 day incubation period at 25° C, mycobacteria were isolated as performed for untreated water samples. Six raw water samples from a single water system collected at monthly intervals yielded 27 *Mycobacterium* spp. isolates by direct recovery. A total of 42 *Mycobacterium* spp. isolates were recovered following protozoa addition. Not only were numbers of isolates higher, but the diversity of isolates greater following the use of phagocytic protozoa to scavenge and concentrate mycobacteria.

Mycobacterium xenopi and closely related organism in the Finnish environment

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We have examined water samples from Finnish streams to evaluate natural reservoirs of environmental mycobacteria. 755 mycobacterial isolates from 53 brook water samples were tested for growth rate, pigment production and growth at 20, 30, 37, 42 and 45°C and also analyzed by gas liquid chromatography (GC). According to these tests and GC profiles we selected three strains for this study (E43, E311 and E347). These strains seemed to be close to *Mycobacterium xenopi*. The type strain of *M. xenopi*, ATCC 19250^T was chosen as the reference strain.

Identification of the strains based mainly on gas liquid chromatography of cellular fatty acids, alcohols and mycolic acid cleavage products. Final identification was done with genetic analysis of ribosomal RNA genes. The entire 16S rRNA gene and the internal transcribed sequence (ITS) between 16S and 23S genes were sequenced and compared to the sequence of the type strain.

The results of these experiments showed that one of the isolates, E341, was similar to *M. xenopi*. To our present knowledge, this is the first report of *M. xenopi* from natural waters. The other two isolates, E43 and E347, were similar to each other but differed from the *M. xenopi* type strain in fatty acid and [illegible] GLC analysis and genetic analysis. This data indicates that these isolates belong to a new mycobacterial species and we propose it to be named *Mycobacterium bohemense* sp. nov.

Atypical micobacteria in environment

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The study of the ecology of mycobacteria is in an early stage compared with that of other microorganisms. In this study we looked for the repartition of mycobacteria in environment.
We used a method first described by Brisou 1982 and adapted by Thorel et al in 1991. This method uses the enzymatic activity of polysaccharases to release mycobacteria from natural environments. We also used an other technique based on treatment with hexadecylpyridinium chlorure (HPC) 0.75 % described by Whipple and Merkal 1983, adapted by Mehmel 1984.
Mycobacteria were isolated from all types of environment samples coming from different areas. Most isolates were obtained at 20°C from biotopes with pH ranging from 3.1 to 8.3 and with moisture percentage between 17 and 83 %. We identified 77 strains of mycobacteria using conventional identification tests : *Mycobacterium kansasii* (1), *M. kansasii* nb pigmented (3), *M. terrae* complex (1), *M. simiae* (2), *M. szulgai* (4), *M. gordonae* (10), *M. malmoense* (13), *M. fortuitum* (7) and *M. chelonae* (36).
This study shows that these mycobacteria were very well adapted to environmental conditions (temperature, moisture, pH). In addition some strains have not exactly the same characteristics of reference strains. These results drive the authors to question about the real meaning of these differences.They underline the fact that the presence of various species of atypical mycobacteria in high numbers in the environment can be an important source of contamination for animals and man.

Mycobacteria in tap water: role in human diseases

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Mycobacteria other than tubercle bacilli are largely present in the environment and their infections are assumed to be acquired from the environment. They frequently contaminate tap water which may be the source for infections. In Europe, *M. gordonae*, *M. xenopi*, and *M. kansasii* represent the predominant species , *M. fortuitum* and *M. chelonae* are less frequently isolated. In USA, *M. avium* is often isolated from tap water whereas such isolation remains rare in Europe. Contact with mycobacteria may lead to colonization of healthy individuals, to pseudo-infection, i.e. contamination of the clinical specimen with exogenous mycobacteria, or to iatrogenic or nosocomial infection. Pseudo-infections may occur during collection of specimens or during treatment of specimens in microbiology laboratories. They lead to diagnostic delays and/or unecessary treatments. Nosocomial infections have been reported and the source of infection usually traced to tap water. Lesions due to iatrogenic infections may be localized or disseminated. They have been related to injections of diverse pharmalocogical substances (vaccines, antibiotics, corticoides, mesotherapy), to invasive diagnosis by endoscopy, to dialysis, to wound treatment, or to surgery (graft, transplantation, cardiac or rachis surgery,...). Their occurrence is usually related to deficiencies in sterilization of water, drug solutions, medical or surgical instruments. Autoclave sterilization has to be strongly recommended.

Role of the integrase on the mycobacteriophage MS6 site-specific recombination event

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Mycobacteriophage Ms6 is a temperate phage of *Mycobacterium smegmatis* that forms stable lysogens by a site-specific integrative mechanism. The integrative cassette has been previously characterized and sequenced.

The integrase protein is required for the integration/excision events of several temperate bacteriophages. To understand further the Ms6 integration/excision events and its regulatory mechanisms the role of the integrase protein was investigated.

In this report we show that the Ms6 *Int* function is absolutely required for the integrative event. Furthermore, we show that in lysogens, the integrase is constitutively produced. The integrase expression in the prophage state is driven by a specific promoter located into a intergenic region between the $tRNA^{Ala}$ gene and the 5' side of the *int* gene. Integrase inactivation by allelic exchanging between prophage DNA and the *int* mutated gene, carried on a suicide vector, led to the curing of the lysogen. The data presented here indicates that the constitutive synthesis of the integrase is associated with the maintenance of Ms6 prophage. Our results also indicates that the mechanisms of Ms6 integration/excision are not identical to those of related temperate phages. *Int* mutants of other temperate bacteriophages such λ and P2 are ineffective in prophage excision since one of the functions required for excision is not supplied. In Ms6, *int* prophage inactivation led to the loss of the prophage which suggests that the integrase is not required for excision. The possibility of controlling the integration and excision of heterologous DNA, using this recombination system, will make the search of gene function, easier and therefore could contribute for the mycobacteria genetic analysis.

Identification of a putative haemolysin gene from *Mycobacterium avium*

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In developed countries organisms belonging to the *Mycobacterium avium* complex (MAC) are the commonest cause of systemic bacterial infections in AIDS patients. In a healthy population, MAC is a rare cause of pulmonary disease that mimics tuberculosis, but in immunocompromised patients it causes life-threatening disseminated infections. What strategies *M. avium* employs to evade being killed by host cells are as yet unknown. Haemolysin have been shown to be important virulence factors in a number of intracellular pathogens, for example *Shigella, Rickettsia* and *Listeria monocytogenes*. It was recently demonstrated that proteins from *M. avium* clinical strains isolated from AIDS patients have haemolytic activity. Furthermore, we have recently cloned, sequenced and characterised haemolysin from *M. tuberculosis* (Wren *et al.* Microbiology 144:May 1998)

In this study we amplified a 321 bp putative haemolysin gene fragment from a clinical isolate of *M. avium*, using degenerate primers designed against conserved regions of previously characterised haemolysin from other bacterial pathogens. Sequence analysis of the *M. avium* clone revealed 77% amino acid identity and 81% similarity with the *M. tuberculosis* counterpart. Preliminary genetic analysis suggests heterogeneity of the putative haemolysin gene among *M. avium* isolates.

Further work is in progress to study the prevalence and the role of the haemolysin homologue in the pathophysiology of *M. avium*.

Characterization of a mycobacteriophage Ms6 promoter region

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A 250 bp mycobacteriophage Ms6 promoter region was detected using a promoter probe vector, carrying an *Escherichia coli lacZ* gene as reporter gene.

The aim of this study was to characterize the genetic elements of this promoter region.

The transcriptional start site, (+1) was determined, using the primer-extension analysis, and the -10 region (TACACT) was located at about 7 bp upstream. We did not find any consensus -35 region sequence.

A leader sequence with 190 bp was found between the transcription start site and the first open reading frame. A second open reading frame was mapped 250 bp downstream. Gene fusions between the first ORF and the *lacZ* reporter gene were carried out to test the promoter's strenght. *M. smegmatis* strains carrying this construction did not show any β-galactosidase activity. This result contrasts with the high level of β-galactosidase activity of pMG1, which does not have the leader sequence. In order to investigate the effect of this leader sequence in the first ORF expression, a new construction was carried out, in which the leader sequence was totally deleted. With this new construction, a high level of β-galactosidase activity was achieved. This leader sequence was subsequently inserted into the plasmid MG1 and a significant decrease of β-galactosidase activity was observed.

Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*

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To characterize the correlation of the mutations in the *pncA* gene encoding pyrazinamidase (PZase) of *Mycobacterium tuberculosis* to a loss of PZase activity and development of pyrazinamide resistance. We determined the association of PZase activity, minimum inhibitory concentrations (MICs), and mutation in the *pncA* gene of *M. tuberculosis* isolated in mostly Asian countries. One hundred thirty-five out of 168 isolates were PZase positive, and 33 were negative. The MICs of PZA at ph 6.0 were over 400 μ g/ml for all 33 PZase-negative isolates , while those of PZase-positive isolates were equal to or less than 200 μ g/ml. Among 33 PZase-negative isolates sequenced, 32(97%) had mutations within the *pncA* gene. A mutation was seen in various regions throughout the *pncA* gene. It was surprised that all three strains of in vitro selected PZA resistant mutation were PZase-positive and showed no change in the *pncA* gene. These results indicate that additional mechanisms may be involved in PZA resistance. No mutations were observed in all 135 PZase-positive *M. tuberculosis* isolates tested, indicating that mutations in the *pncA* gene could be involved in the loss of PZase activity. These results suggest that sequencing analysis of the *pncA* gene should provide rapid diagnosis of PZA resistant clinical isolates of *M. tuberculosis*.

Investigating mycobacteriophage DS6A infection

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Mycobacteriophage DS6A has been shown to infect all *M. tuberculosis* strains and *M. bovis BCG*, but does not infect *M. smegmatis*. In order to identify the genes responsible for conferring susceptibility to DS6A infection we have used a cosmid library of *M. tuberculosis* H37Rv transformed into *M. smegmatis*. Two cosmid-containing clones of *M. smegmatis* (1E1 and 1H1) were found to be sensitive to phage infection. A region of approximately 7kb of cosmid 1E1 was found to be sufficient to confer DS6A sensitivity in *M. smegmatis*.

Phages passaged through *M. smegmatis* 1E1 or 1H1 were then also able to infect wild type *M. smegmatis*. This altered phage phenotype is retained after a further passage through *M. tuberculosis*. We have also found several variants of the phage which were able to infect wild type *M. smegmatis* demonstrating that there is quite a high frequency of phage adaptation/mutation to host strains. However, some phage variants produce unusual patterns of plaquing indicating more complex changes to the infection cycle.

An improved understanding of the genetics of phage infection may facilitate the construction of stable mutants with defined susceptibility to phage infection. These in turn may provide useful tools for diagnostic and typing studies.

Determination of an internal origin of replication in a mycobacterial linear plasmid: pCLP from *M. celatum*

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The presence of extrachromosomal elements in *M.senopi*, *M.celatum*, *M.avium* and *M.branderi* that migrated in pulsed field gel electrophoresis (PFGE) like linear molecules was recently described. The susceptibility of these elements to exonucleases and the presence of covalently bound proteins suggested an invertron-like structure (Picardeau and Vincent, 1997 J.Bacteriol. 179: 2753-2756, 1998 Microbiol. in press.).
The 25 kb linear plasmid DNA of *M.celatum* strain 4, designated pCLP, was analysed. In a subcloning experiment, an internal fragment of 5kb of pCLP was introduced into a pUC19 derivative resulting in the plasmid pCL4R. The plasmid pCL4R was maintained in *M.smegmatis* mc² 155 and in *M.bovis* BCG. Furthermore the results of RFLP analysis showed that the plasmid pCL4R is not integrated in the chromosome and thus is a replicative plasmid. The new replicon is compatible with pAL5000 and with pJAZ42 (pJAZ38 derivative) circular plasmid derivatives, suggesting a new class of plasmid. pCL4R was also found to be stably maintained in mycobacteria in the absence of selection pressure. The relative plasmid copy number, calculated by single-cell resistance to kanamycin, was determined to be 1.
All of these findings make pCL4R a good candidate for further genetic studies of mycobacteria.

Identification of a mycobacteriophage Ms6 DNA gene that confers inhibition to superinfection

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Mycobacteriophages are important tools for the development of genetic systems for Mycobacteria. Ms6 is a temperate phage that infects *Mycobacterium smegmatis* and forms stable lysogens that are immune to superinfection.
A 4,8kb *Bgl* II restriction segment of Ms6 DNA was sequenced and demonstrated to have a site specific recombination locus of the phage. We also observed that *M.smegmatis* recombinant strains carrying this fragment were resistant to superinfection.
The aim of this study was to identify the genetic determinants of the inhibition to Ms6 superinfection.
Smaller fragments of the 4,8kb *Bgl* II segment were subcloned into a shuttle plasmid, pRRJ (Monica Rames et al, 1990), and tested for their ability to confer inhibition to Ms6 superinfection in *M. smegmatis*.
The capacity to inhibit a superinfection to Ms6 was associated to a 978bp *Sspl-Scal* restriction fragment which contains a open reading frame that is 453bp in lenght and encodes a 151 aminoacid protein.
In order to confirm that property we subcloned this gene in a expression integrative vector derived from phage D29. The *Mycobacterium smegmatis* strains containing these integrated plasmid became resistant to Ms6 superinfection.
In conclusion we demonstrate that this gene is responsible for the inhibition to Ms6 superinfection.
The development of selectable genetic markers alternative to the antibiotic-resistance genes are important tools for the mycobacterial genetic manipulation.

Constrution of mycobacteriophage D29–derived vectors for genetic manipulation of mycobacteria

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BCG has large potentiality and advantages as the basis for the construction of recombinant vaccines against multiple diseases. Vectors for the genetic manipulation of mycobacteria are also essential for the study of the biology and pathogenesis of those bacteria and will lead to significant advances in therapy and diagnosis.

This work describes the construction of two vectors for expressing different genes in mycobacteria. These vectors have the integration region of mycobacteriophage D29 to promote the integration of vector in the chromosome of mycobacteria in the 5' or 3' end of the gene of green fluorescent protein (GFP) of jellyfish Aquoria victoria. This gene is under the control of the kanamycin promoter of Tn903 that is well expressed in mycobacteria and is there in substitution for the antibiotic marker. We amplified the heat shock promotor of Mycobacterium tuberculosis and ligated it in a multiple cloning site. To terminate transcription we introduced rrnB1 terminator of E.coli.

DNA site-specific integration into BCG chromossome by transcomplementation

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BCG, an avirulent strain of *M. bovis* developed by Calmette and Guérin, offers some advantages as delivery vehicle of foreign antigens. In recent years genetic tools for mycobacteria were developed allowing the expression of multiple protective antigens of different pathogens in BCG. However, Plasmid vectors are not very stable in absence of a continuous selection. To increase the stability of the recombinant strains we develop a DNA integration process based on the site-specific integration system of temperate mycobacteriophage Ms6 isolated and characterized in our laboratory.

In this study a new procedure of DNA integration is disclosed that consists in providing the integrase gene on a suicide vector, that transiently produces the integrase required for the insertion of a second plasmid vector containing the Ms6 *attP* region and the foreign gene or genes to be expressed. The recombinant bacteria obtained are very stable as phage excision or integration functions are not present. With this procedure we achieved 98% of cells that conserve the vector integrated in their genomes after 50 generations in a non selective medium. In contrast, only 50 % of survivors where obtained using an extrachromosomal plasmid vector.